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COLIFORMS IN COTTAGE CHEESE
AND THE RELIABILITY OF SELECTIVE MEDIA
FOR THEIR ENUMERATION AFTER HEAT TREATMENT

by



Lawrence Albert Roth

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
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The undersigned certify that they have read,
and recommend to the Faculty of Graduate Studies for
acceptance, a thesis entitled COLIFORMS IN COTTAGE
CHEESE AND THE RELIABILITY OF SELECTIVE MEDIA FOR
THEIR ENUMERATION AFTER HEAT TREATMENT submitted by
Lawrence Albert Roth in partial fulfilment of the
requirements for the degree of Master of Science.

ABSTRACT

The high incidence of coliforms in cottage cheese justified an investigation into their source and occurrence. Also, it was attempted to determine the types and sources of microorganisms which were most detrimental to shelf life. It appeared that yeasts and molds represented a major group of spoilage organisms in cottage cheese from four Edmonton dairies. It was concluded that lack of sound sanitation programs was probably the major factor responsible for spoilage microorganism and coliform contamination.

Coliforms were generally destroyed by the cooking process in cottage cheese manufacture and yet coliforms were frequently isolated from the curd later in the process. This was primarily attributed to recontamination during curd handling, but it was also questioned whether this disappearance and subsequent reappearance of coliforms could be the result of heat "injury" and "recovery" which had been established for Gram-positive bacteria. Preliminary experiments showed that heat injured cells displayed an increased sensitivity to selective agents making enumeration of these cells inaccurate with selective media. To investigate the reliability of enumeration of Gram-negative organisms a study was undertaken to determine the occurrence of the heat "injury" and "recovery" phenomenon for Escherichia coli and Salmonella typhimurium. It was observed that sublethally heated cultures of these organisms

yielded much lower counts when plated on selective media compared to counts on nutrient agar, indicating heat injury.

The reliability of liquid media for use in determining the coliform content and for salmonellae enrichment was also investigated. While the solid and liquid media recommended for enumeration of coliforms, appeared to give accurate counts of unheated cultures of two strains of E. coli, these media proved unsatisfactory for the enumeration of sublethally heated cells of these two strains. Similar inhibition of growth in selective media and enrichment broths was observed for sublethally heated Salmonella typhimurium cells. These results indicated the need for cautious interpretations when enumerating stressed coliforms and salmonellae on the selective media recommended.

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COLIFORMS IN COTTAGE CHEESE

INTRODUCTION AND LITERATURE REVIEW

It is generally considered that the problem of coliforms in dairy products can be controlled by proper processing procedure and careful sanitation. Yet in 1967, 40% of the samples entered in the Alberta Dairymen's Association cottage cheese competition were rejected because of coliform counts exceeding the Food and Drug standard of not more than 10 coliforms/g (Department of National Health and Welfare, 1954). In the same year, 51.8% of the samples examined by the Alberta Dairy Branch Laboratory, Edmonton, failed to meet this standard. Although the situation appeared to improve in 1968, the Dairy Branch Laboratory reported that 27.6% of the cottage cheese samples tested contained more than 10 coliforms/g (Kadis, personal communication).

A survey of cottage cheese in Kansas, U. S. A. showed 71.2% of 142 samples contained more than 10 coliforms/g (Martin, Foltz and Rutz, 1960). Overcast, Skean and Britton (1961) in a survey in Tennessee observed a wide variation in coliform content of cottage cheese with counts ranging from <1 to 715,000/g with a median of 422/g. The high coliform counts in commercial cheese prompted these researchers to manufacture cottage cheese to determine what level of coliform

contamination could be expected. Of 64 lots manufactured using normal procedures, only three samples had an initial coliform count exceeding 10/g. Earlier, Overcast and Britton (1959) reported that using acidified and chlorinated wash water they were able to keep the coliform count of fresh cottage cheese and 11-day stored cottage cheese below 10/g. When a Michigan dairy mechanized the cottage cheese packaging operations the percentage of samples containing coliforms dropped from 64% to 33% (Lyons and Mallmann, 1954). These workers felt that the mechanization of all steps in the processing and packaging of cottage cheese would do much to eliminate coliform contamination. Harmon and Smith (1956) observed that for 17 vats of cottage cheese in 12 dairies the pasteurized skimmilk usually contained coliforms; the counts rose during the setting of the curd, but subsequently dropped during the cooking process. In the same study contaminated dressing was observed as a major source of coliforms.

The efficacy of various cooking procedures has been studied by Collins (1961). A final cooking temperature of 54.9°C (130°F) maintained for 18 min proved satisfactory for destroying the Escherichia coli strains used. Although the cooking process appeared to destroy all coliforms, if large numbers of coliforms were present at cutting time their presence was detected on storage (Tuckey, 1959). The production of coliform-free cottage cheese is important since small numbers of these organisms in the finished product are able to multiply during storage. Bonner and Harmon (1957) noted growth of an E. coli strain isolated from cottage cheese at a temperature as low

as 3°C. The same organism showed maximum growth near neutrality, but showed slight growth at a pH as low as 4.4, indicating that coliforms could multiply in cottage cheese under storage conditions. Skelton and Harmon (1964) also reported growth of four strains of E. coli in cottage cheese stored at 10°C. As a possible method of reducing the growth of coliforms in stored cottage cheese, Mather and Babel (1959) suggested the use of a special creaming mixture containing Streptococcus citrovorus. They observed a marked inhibition of coliform growth in cottage cheese creamed with this dressing and stored at 7.2°C for 13 days.

Coliforms in cottage cheese are considered to result from poor sanitary practices in the dairy and most researchers have suggested greater care with equipment sanitation, handling of raw materials and pasteurization and cooking processes. Accordingly, the inability of the cottage cheese producers of Alberta to meet the Food and Drug standard indicated that a study was desirable.

It was first decided to investigate the occurrence and source of coliforms in commercially manufactured cottage cheese. After this survey had commenced, dairy managers expressed a concern about the shelf life of their product, which was estimated at two weeks, not long enough to cover marketing and household storage time. The organisms shown to be most detrimental to shelf life were found to be Gram-negative psychrophiles (Martin, Foltz and Rutz, 1960; Cannon, 1965). A large increase (<10 to 5,000/g) in the psychrophilic count of cottage cheese stored at 4.4°C for 14 days was reported by

Tuckey (1959). Also Overcast and Skean (1958) observed large increases in the psychrophilic and yeast and mold counts of cottage cheese stored at 4.4°C for 15 days. Much of the slimy, gelatinous defects noted on storage of cottage cheese was shown to be caused by growth of Pseudomonas fragi, Pseudomonas viscosa, and Alcaligenes metalcaligenes (Parker, Smith and Elliker, 1951). While researchers generally agreed that the shelf life of cottage cheese was shortened by the presence and growth of psychrophilic bacteria, they considered that the numbers of psychrophiles could be controlled by careful sanitation and low storage temperature.

While it was planned originally to investigate the source and occurrence of coliforms in cottage cheese, the study was later expanded to assess the microbiological quality of cottage cheese being produced in Edmonton.

MATERIALS AND METHODS

Sampling

The samples for this study were obtained from four representative Edmonton dairies. The cheesemakers were provided with sterile sample jars, dippers, spoons, and an information sheet (Figure 1) for each batch of cheese manufactured, and instructions for taking bacteriological samples. The samples were collected from the dairy shortly after packing commenced and were transported on ice to the laboratory. In the laboratory the samples were blended [11 g with 99 ml of 2% (w/v) sodium citrate] in a Waring Blendor jar for 3 min. Subsequent dilutions were made into 1/4 strength Ringer's solution (Oxoid). The liquid samples were also diluted with 1/4 strength Ringer's solution.

Bacteriological tests

Standard plate count. This was done using Difco plate count agar and incubation at 32°C for 48 ± 3 hr.

Coliform count. Coliforms were enumerated by plating the cheese samples with Difco violet red bile agar and incubating at 32°C for 24 ± 2 hr. The solidified poured violet red bile agar plates were overlaid with an additional 3 to 4 ml of plating medium before incubation to prevent surface colony formation.

Psychrophilic count. For this poured plates of Difco plate count agar were incubated at 4.4°C for 10 days.

Procedure for obtaining samples during
cottage cheese manufacture

PLEASE STORE ALL SAMPLES IN COOLER

- | | |
|--|--------------------------------------|
| 1. Sample skimmilk from cheese vat after starter and rennet have been added. (Wrapped dippers are pre-sterilized. Remove foil without touching wrapped surface with hands. Care should be taken not to touch dipper with anything prior to sampling. Jar should not be left open for extended periods and lids should be placed top down when laying down to avoid contamination.) | Time of sampling_____ |
| | Time of set_____ |
| | Per cent solids_____ |
| 2. Sample starter. | |
| 3. Sample just after cutting. | |
| | Cutting time_____ |
| | % Titratable acidity_____ |
| 4. Sample curd just after draining. | |
| | Time of sampling_____ |
| | Final cooking temperature_____ |
| 5. Sample final washed curd. | |
| | Time of sampling_____ |
| 6. Sample creaming mixture. | |
| | Date of manufacture of dressing_____ |
| 7. Sample creamed curd prior to placing in packer hopper. | |
| | Time of sampling_____ |
| 8. One final packed carton taken shortly after the first few packages have been filled. | |
| | Time of sampling_____ |

Figure 1. Sampling instructions and information sheet.

Yeast and mold count. Poured plates of Difco potato dextrose agar, acidified to pH 3.5 with tartaric acid were prepared and incubated at room temperature for three to five days.

Storage tests

After aseptic removal of a sample from the final packed product, the carton of cheese was stored at 4.4°C. The microbial counts were repeated on the stored cartons of cheese at approximately weekly intervals for three weeks.

Phosphatase test

With samples from Dairy D, where it seemed possible that the skimmilk may not have been adequately pasteurized, a phosphatase test was carried out on the skimmilk sample taken from the cheese vat. The test used was the Scharer 1 (Rapid) Method given in Standard Methods for the Examination of Dairy Products (1960).

Swab examinations

At Dairies B and D some of the cottage cheese processing equipment was swabbed to determine presence of coliforms. The swabbing equipment consisted of 12-gauge stainless steel wires, 35.5 cm long, which had 15 cm of 2-in unmedicated ribbon gauze wound around the rod and secured with thread. The swabs were placed in 25 x 250 mm test tubes containing 25 ml of buffered rinse solution described in Standard Methods (1960). Where possible an area of 1 ft² was swabbed in such a manner that the area was treated twice. The swabs were

returned to the rinse solution and transported on ice to the laboratory. After vigorous agitation, 2 ml of the rinse solution were plated into violet red bile agar (Difco) and incubated for 24 ± 2 hr.

Bacteriological groups

To determine changes in the incidence of different types of microorganisms during the storage of the cheese, the procedure used by Jackson (1963) was adopted. Standard plate count plates which contained between 30 and 300 colonies were used to obtain the 20 colonies. These colonies were selected at random by drawing a 4 x 5 grid on the back of the plate, and picking those colonies closest to each of the 20 intersections. The colonies were inoculated into tryptic soy broth tubes and incubated at 32°C for 24 hr. The cultures were then streaked on plate count agar plates and incubated for 24 hr at 32°C. Catalase production by the cultures was detected using a 3% (v/v) hydrogen peroxide solution. The cultures were Gram-stained and examined microscopically. Catalase positive Gram-negative rods were inoculated into MacConkey's broth and incubated at 32°C for 24 hr for the detection of coliforms. The organisms were placed into one of the groups shown in Figure 2.

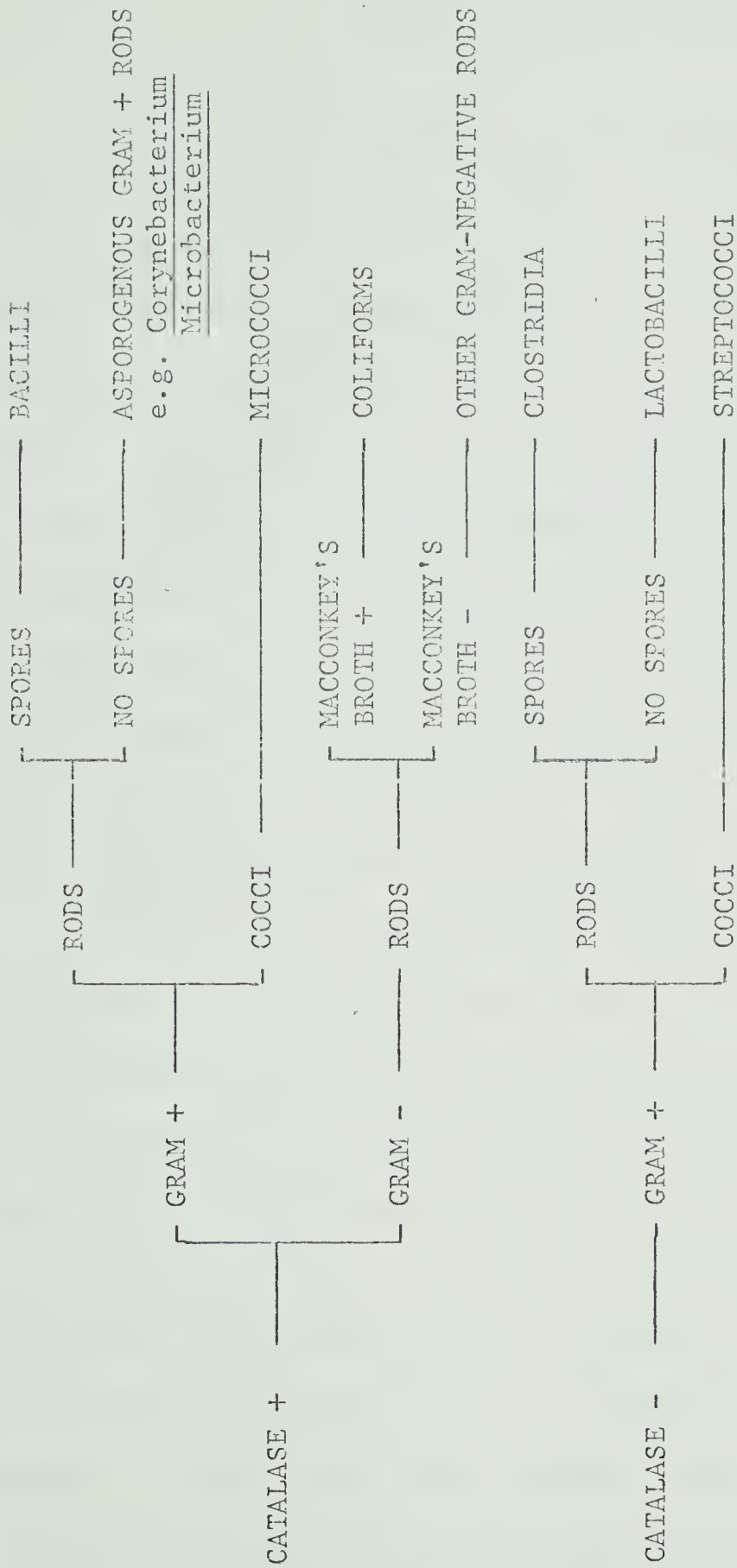


Figure 2. Scheme for identification of the main types of bacteria (Jackson, 1963; after Carreira et al., 1955).

RESULTS AND DISCUSSION

The data obtained for the microbiological quality of the cheeses are difficult to represent, because of the great variability between methods of manufacture and the degree of sanitation practiced at each dairy. However, a generalized pattern has been prepared for each dairy, based on the modal values. These data are presented in Tables 1 to 4, and the data for the individual cheeses manufactured at each dairy are included in Appendices 1 to 7. The incidence of the different groups of microorganisms observed in 21 samples of cottage cheese after manufacture and at subsequent times during storage are shown in Table 5. Results for only three dairies are shown in Tables 4 and 5 as the study was expanded to include shelf life after the coliform study at one dairy was almost complete.

Of the cottage cheese samples examined, 48% contained more than the Food and Drug Administration limit of not more than 10 coliforms/g at the end of manufacture. The results presented in Table 1 indicate the apparent source of the coliforms in the manufacturing procedure. Both Dairies A and D had direct recontamination of their product by contaminated dressing. In Dairy A, failures in the cleaning-in-place procedure of the pipeline used to transport cream from the separator to the dressing tank resulted in a coliform-contaminated creaming mixture. If this fault were corrected this dairy should be capable of producing cheese which would satisfy the coliform standard.

Table 1 Changes in the modal coliform content*
of cottage cheese during manufacture

Dairy	Number of Samples	Skim Milk	Starter	Cutting	Draining	Dry Curd	Dressing	Creamed Curd	Packed Product
A	9	-	-	-	-	-	+	+	+
B	10	-	-	-	-	-	-	-	±
C	14	++	-	++	-	-	-	±	+
D	16	±	-	+	-	-	±	-	+

*Coliform count (/ml or /g): - = 0, ± = 1 to 10, + = 11 to 100, etc.

Table 2 Changes in the modal bacterial content
of cottage cheese during manufacture

Dairy	Number of Samples	Standard plate count /ml or /g						
		Skim Milk	Cutting	Draining	Dry Curd	Dressing	Creamed Curd	Product
A*	6	10^3	10^5	10^4	10^3	10^3	10^3	10^3
B	10	10^6	10^7	10^5	10^5	10^3	10^5	10^5
C	14	10^7	10^8	10^3	10^4	10^3	10^4	10^4
D	4	10^5	10^6	10^5	10^4	10^6	10^4	10^4

*Skimmilk samples taken prior to addition of starter.

Table 3 Changes in the modal psychrophilic microbial content*
of cottage cheese curing manufacture

	Number of Dairy Samples	Skim Milk	Cutting	Draining	Dry Curd	Dressing	Creamed Curd	Packed Product
A	6	±	±	±	±	±	±	±
B	10	±	±	±	±	±	+	+
C	11	±	±	±	±	±	+	±
D	4	+++	++	++	+++	++	+++	+++

*Psychrophile count (/ml or /g): ± = 1 to 10, + = 11 to 100, etc.

Table 4 Changes in the predominant types of microorganisms
during storage of cottage cheese at 4.4°C *

Storage (days)	Dairy	Coli- forms	Standard Plate Count	Psychro- philes	Yeasts, Molds
0	A	+	10^3	+	10^3
	B	+	10^5	+	10^2
	C	+	10^4	+	10^2
7 - 10	A	+	10^5	+++	10^3
	B	+	10^3	+++	10^5
	C	+	10^4	+	10^2
14 - 17	A	+	10^7	+++++	10^5
	B	+	10^6	++++	10^7
	C	+	10^6	++++	10^4

*Count (/ml or /g): $\pm$ = 1 to 10, + = 11 to 100, ++ = 101 to 1000, etc.

Table 5 Changes in the incidence of different types of microorganisms in cottage cheese during storage at 4.4°C

[Percentage (and numbers) of Microorganisms]

Type of Organism	At Packing		6 - 15 Days		16 Days	
Asporogenous Gm + Rods	28.1	(80)	48.2	(110)	17.9	(40)
Bacilli	15.8	(45)	3.5	(8)	4.5	(10)
Micrococci	17.2	(49)	11.4	(26)	8.9	(20)
Gram - Rods	15.8	(45)	18.0	(41)	10.7	(24)
Coliforms	0.7	(2)	0.4	(1)	0.4	(1)
Lactobacilli	7.7	(22)	4.4	(10)	-	-
Streptococci	10.9	(31)	5.7	(13)	0.9	(2)
Yeast	0.7	(2)	7.9	(18)	56.7	(127)
<u>Sarcina</u>	3.2	(9)	0.4	(1)	-	-
Total		(285)		(228)		(224)

However, with Dairy D, even though the coliforms were absent in 0.1 g of final washed curd, the improvement of the dressing alone would probably not have eliminated coliforms entirely. The general hygienic conditions under which the cheese was produced at this dairy were unsatisfactory and a large proportion of the skimmilk samples contained coliforms, indicating post-pasteurization contamination and/or improper pasteurization. Swabs from the cheese vat of Dairy D showed no coliforms on the vat surface, yet swabs of the pipe from the pasteurizer to the cheese vat showed 400 coliforms/ft². Phosphatase tests on the skimmilk proved negative and therefore at this dairy it was assumed that unsanitary equipment, i.e. transfer pump and pipe, was the main cause of post-pasteurization contamination. The cooking temperatures at Dairy D also varied over a wide range [44.4°C to 56.6°C (112°F to 134°F)], and the final cooking temperature was not usually maintained longer than 10 min. The lower cooking temperatures could account for the coliforms surviving cooking and being present in the final product.

With Dairy B the samples were generally free of coliforms with the exception of the final product, indicating that contamination occurred during packing. The packer often broke down and the subsequent repair operation was probably responsible for some contamination.

Dairies C and D appeared to suffer from the same problem, lack of good hygienic conditions, and the numerous sources of coliforms resulted in a contaminated product throughout the process. To correct

the coliform problem at these dairies, sanitation and product handling would have to be drastically improved, and this would involve some major equipment and building improvements.

No Food and Drug standard exists for the standard plate count of cottage cheese. Although the generally accepted limit for pasteurized milk is as high as 50,000 organisms/ml, with the added heat treatment of the cooking process in cottage cheese manufacture, experience has shown that it is reasonable to expect counts of $<10,000$ micro-organisms/g in the finished product. It is generally accepted that the cooking process is severe enough to destroy the starter organisms. In this study 70% of the samples had a standard plate count $>10,000/\text{g}$. Kadis (personal communication) reported that over the two-year period, 1967 to 1968, 42.4% of the cottage cheese samples tested at the Dairy Branch Laboratory, Edmonton, had a standard plate count $>10,000/\text{g}$. The higher result obtained in this study probably results from the fact that the majority of the samples were collected during summer months when more care would be required during manufacture to obtain counts $<10,000/\text{g}$.

The standard plate counts of the packed product from Dairies C and D were appreciably higher than those of Dairy A (Table 2), which again implied lack of sound sanitation at these two dairies. The coliform content and the high plate count observed for the dressing of Dairy D indicated a need for improvement in the handling of the creaming mixture. The high plate count on the packed product from

Dairy B could have been due to a strain of starter which was resistant to the cooking temperatures, but there is no present experimental evidence to support this.

Although it is considered that manufacturers should strive to produce a cheese of $<10,000$ organisms/g, there was no apparent relationship between colony count and shelf life, because the numbers of organisms growing at 32°C at packing bore no close relationship to the number of psychrophilic organisms that could grow during the storage time at the typical storage temperature of the product (4.4°C).

The large number of psychrophiles observed for Dairy D during manufacture indicate the need for sanitation improvements (Table 3). According to Harmon (1963) the psychrophilic bacteria growing in cheese are predominately Gram-negative rods. During the first week of storage there was only a slight increase in the numbers of Gram-negative rods expressed as a proportion of the total population growing at 32°C (Table 5). However, after 16 days' storage, the slight decrease in numbers of the Gram-negative rods coupled with the high increase in the number of yeast cells resulted in a decrease in the percentage of Gram-negative rods in the total population. The number of Gram-negative rods shown in Table 5 does not give the total number of Gram-negative psychrophiles present, since colonies were obtained from plates used in the standard plate count incubated at 32°C . Table 4 shows marked increases of psychrophiles during storage and although some organisms enumerated by the psychrophilic count were probably yeasts and molds no doubt some were Gram-negative rods.

The dairies examined in this study had cheese vats and equipment vulnerable to air-borne contaminants, as from pipes and fittings over large, open processing areas. Yeast and mold counts, which were determined only on the final product, were generally low in number at the end of manufacture (Tables 4 and 5). However, during storage they showed a dramatic increase from 0.7% of the initial population to 56.7% of the microbial population in the product stored for 16 days. As yeasts and molds grow over a lower pH range than bacteria it is not surprising that yeasts appear to be a major group of microorganisms limiting shelf life of the cottage cheese of Edmonton during storage.

When the titratable acidity at cutting was low, a higher final cooking temperature was required to firm the curd (Table 6, Appendix 8). The cooking process is an essential procedure in firming the curd, but it has the added advantage that it destroys large numbers of microorganisms. Cheese processors must maintain a low titratable acidity and corresponding final cooking temperature balance which will produce the best combination of texture and microbial control.

Table 6 Mean per cent titratable acidity (expressed as per cent lactic acid) at cutting and final cooking temperature of the samples obtained from four commercial dairies in Edmonton

Dairy	Titratable Acidity, %	Final Cooking Temperature
A	0.485	58.7°C (137.7°F)
B	0.505	51.2°C (124.1°F)
C	0.554	51.7°C (125.0°F)
D	0.564	50.2°C (122.4°F)

CONCLUSIONS

None of the dairies had any precise figures on shelf life, and unfortunately there is no objective test to determine this. The staff of Dairy A felt their cheese exhibited a shelf life no longer than cheese from the other dairies and yet in this study Dairy A produced cheese of the best general microbiological quality. The short shelf life (two weeks) they have estimated for their cheese indicated that further improvements in manufacture were necessary. Possibly the large, open processing area of Dairy A accounted for some yeast and mold contamination, and modification to a smaller cheese room might reduce the occurrence of these organisms and enhance the keeping quality of their cheese. Minor improvements in sanitation might be sufficient to alleviate the coliform problem in this dairy.

It is highly probable that improvements in sanitation of the cheese manufacturing section of Dairy B would have reduced the coliform counts to meet the Food and Drug standards. At the same time this would probably have reduced the number of spoilage microorganisms and thus improved the keeping quality of the cottage cheese. A smaller cheese room (one which could be more easily disinfected) as well as some equipment renewal would be helpful in producing cheese of good bacteriological quality.

Dairies C and D required more attention to sanitary practices to reduce the coliform count and increase the keeping quality of their cheeses. These dairies are located in old buildings, making it

difficult to maintain good sanitation of cheese-processing rooms. Major renovations would be helpful but might not be economically feasible.

Yeasts and molds appeared to be major spoilage organisms in the cottage cheese examined; this was in agreement with the observations of Overcast and Skean (1958). However, Harper and Randolph (1965) noted that yeasts and molds could not be considered as prime cottage cheese spoilage microorganisms in Ohio. Olson and Ball (1957) and Geminder (1959) showed that potassium sorbate was most effective for reducing spoilage of cottage cheese due to yeasts and molds. Huang (1969) recently noted that potassium sorbate effectively increased the storage life of cottage cheese of an Edmonton dairy. These findings indicated the possibility that yeasts and molds are important spoilage organisms of Edmonton cottage cheese.

In general it was evident that, in all the dairies of this study, sanitation of rooms and equipment was not given enough attention. It appeared that the industry was searching for a substitute for good sanitation and hygiene. Dairies which exhibited poor sanitation practices tended to rely on the future possibility of additives for improved shelf life. As much as additives are effective in increasing shelf life (Bradley and Harmon, 1962; Olson and Ball, 1957) they should not be used as substitutes for good sanitation. The lack of appreciation of sanitation was possibly due to the lack of control applied to the dairies by government agencies, as evidenced by the

fact that approximately 50% of the cottage cheese retailed in Alberta does not comply with the Food and Drug coliform standard.

To improve the situation, qualified personnel must be employed in the quality control divisions of the dairies. Dairy management did not appear willing to hire adequately trained employees. Furthermore, the communications and understanding among management, quality control, and plant personnel was less than satisfactory. Under these circumstances the quality of the product suffered because problems in the plant were not brought to the attention of management and plant employees were not capable of correcting the problems without supervision. One of the most difficult aspects of the sanitary production of cottage cheese is the training of personnel. The quality of the cottage cheese is almost entirely dependant upon the cheesemaker, and it is necessary that he understand fully the principles of good sanitation in the dairy.

The cooking process of cottage cheese manufacture is capable of destroying large numbers of microorganisms (Emmons, 1963), and yet three of the dairies of this study did not use cooking procedures which afforded good microbial control. Collins (1961) has shown that cooking procedures which did not have a maximum temperature of 54.9°C (130°F) held for 18 min were not effective in destroying psychrophiles and starter bacteria. Some of the dairies were not achieving a final cooking temperature this high and it is recommended that their manufacturing process be changed to include a final cooking temperature in excess of 54.9°C (130°F). To achieve this and still maintain the

desired firmness of curd, the curd must be cut at a lower titratable acidity. Other variable factors in the manufacturing process did not permit a definite titratable acidity/final cooking temperature relationship to be defined from the data obtained in the present studies.

As much as poor sanitation appeared to be the major factor responsible for the coliforms in the cottage cheese of the Edmonton dairies, another factor may be responsible for their occurrence in the final product. Table 7 shows some selected examples of the changes in the coliform counts during manufacture. In these cheese makings the trend of coliforms present at cutting and subsequently destroyed by the cooking process was evident. At some stage after the draining period coliforms reappeared as contaminants in the final product, although the dressings were free of coliforms. This is generally accepted as post-cooking contamination from handling the product. Alternatively, these results suggested the possibility that thermal injury and subsequent recovery of the coliforms occurred. Such injury and recovery phenomena have been demonstrated with sublethal heat treatments of Staphylococcus aureus (Stiles and Witter, 1965) and Streptococcus faecalis (Clark, Witter and Ordal, 1968). It seemed possible that the sublethal heat treatment applied in cottage cheese manufacture during cooking might give rise to a similar phenomenon with the coliforms. It was decided to attempt to illustrate the possibility using strains of E. coli. The next section of this thesis is devoted to the heat injury and recovery of E. coli and Salmonella typhimurium.

Table 7 Selected examples of changes in the coliform content*
of cottage cheese during manufacture

Sample	Skim Milk	Cutting	Draining	Dry Curd	Dressing	Creamed Curd	Packed Product
C3	+	++	-	-	-	+	+
C4	++	++	±	±	-	+	++
C5	++	++	-	-	-	-	+
C7	++	+	-	-	-	-	+
C12	+	+	-	-	-	±	+
C13	++	++	-	±	-	+	+
D4	-	±	-	-	-	-	±
D10	±	+	-	-	-	-	+

*Coliform count (/ml or /g): - = 0, ± = 1 to 10, + = 11 to 100, etc.

HEAT INJURY AND RECOVERY

INTRODUCTION AND LITERATURE REVIEW

For the purpose of this section it is considered desirable to define "injury" and "recovery."

"Injury" represents the loss of some characteristic of the parent culture, which restricts the growth of the injured cells when this specific characteristic is challenged, but from which the injured cells are able to recover.

"Recovery" represents the repair of the damage responsible for injury, enabling previously injured cells to grow normally.

These conditions of injury and recovery are illustrated by Staphylococcus aureus when sublethally-heated cells exhibit an increased sensitivity to salt (heat injury) and are able to regain their salt tolerance when exposed to suitable conditions.

It has been generally observed that bacteria surviving heat exhibit an extended or lengthened lag phase upon provision of optimal growth conditions. Hershey (1939) observed a progressively lengthened period of latency for Escherichia coli cultures as the temperature

of heat treatment was increased. He noted a 1.6-hr lag after heating bacterial cells for 15 min at 51°C. When the temperature was increased to 56°C, a 7.9-hr lag period resulted. Extended lag periods have also been shown for sublethally-heated Staph. aureus (Jackson and Woodbine, 1963), Streptococcus faecalis (Clark, Witter and Ordal, 1968), Pseudomonas fluorescens (Lawton and Nelson, 1955) and a Micrococcus strain (Kaufmann et al., 1959). The extended lag phases were usually demonstrated by incubating the heat-treated cells in a broth and periodically enumerating the viable cells in an agar medium. Strange and Shon (1964), plating sublethally-heated cells of Aerobacter aerogenes, noted that an extended lag phase was probably the cause of the delayed appearance of colonies on agar media. They also noted marked differences in colony size at specific incubation times. This indicates the possibility of a variable effect of the sublethal heat treatment on the cells. Heated cells which exhibited the prolonged lag phase showed normal growth once they began to divide. Thus it appeared that the sublethal heating process caused cellular injury which had to be repaired before normal growth resumed. This cell injury has been evidenced by other factors such as more exacting nutrient requirements, narrower temperature and pH ranges permitting growth, reduced oxygen consumption, and an increase in sensitivity to inhibitors and selective agents. The extent of cell injury resulting from adverse conditions may be estimated by observing any one of these parameters mentioned.

Nelson (1943) compared four agar media for their ability to support growth of pure cultures of bacteria which had been sublethally heated. Because the complex natural medium, beef infusion agar, gave higher counts of heated bacterial cultures than a synthetic medium, he concluded that bacteria which have been subjected to partially lethal heat treatments were more exacting in their nutritional requirements. Nelson suggested that this factor be considered when selecting media to enumerate bacteria in heated foods and in experiments concerned with the effect of heat upon microorganisms. In later experiments, Nelson (1944) observed that the addition of tryptone to a synthetic medium resulted in large increases in the number of bacterial cells that had been heat treated. Russell and Harries (1968) showed that the addition of yeast extract (1% w/v) to nutrient agar caused an increase in the count of heat-treated E. coli from $2.5 \times 10^3/\text{ml}$ to $1.5 \times 10^4/\text{ml}$. They added the five vitamins present in yeast extract singly and in combination to a synthetic medium but observed no increase in growth over the control, indicating that the increase in growth was not due to the vitamins in the yeast extract. The addition of all the amino acids present in yeast extract to the synthetic medium did not enhance the growth of the heated E. coli to the same extent as synthetic agar containing yeast extract. However, the omission of methionine, lysine, and threonine from the synthetic medium did result in some decrease in survival. Russell and Harries, using the replica plating technique, showed that no stable auxotrophic mutants of E. coli were found during sublethal heating.

Heather and Van der Zant (1957a) reported that higher counts of heat-treated Ps. fluorescens were obtained in nutrient and yeast extract agar than on a simple synthetic medium, thus indicating that this psychrophile appeared to exhibit more exacting nutrient requirements after sublethal heat treatment. Lawton and Nelson (1955) observed higher counts of heat-treated Ps. fluorescens on plate count agar than on tryptone glucose extract agar. This also indicates the more exacting nutrient requirements of sublethal heating, since plate count agar was designed to substitute for tryptone glucose extract agar with skimmilk added.

Nelson (1944) showed that the presence of added nutrients in the medium was not the only controlling factor in determining the ability of heat-treated bacteria to multiply since a medium supplemented with thioglycollic acid yielded higher counts of heat-treated bacteria. Harries and Russell (1966) noted that a larger number of E. coli cells survived heat treatment when enumerated by the pour plate technique rather than by the surface plate technique, suggesting that lowered oxygen availability was desirable.

Heating a culture of E. coli at 52.5°C for 15 min depressed oxygen consumption about 90% and yet did not kill any of the cells (Hershey, 1939). The decrease in oxygen consumption was considered to indicate some form of cell injury which did not reduce cell viability.

Heather and Van der Zant (1957b) showed that heat-treated psychrophiles grew in a more limited temperature range than did

unheated cells and were more sensitive to changes in the pH of the plating medium. Nelson (1956) reported maximum E. coli counts after sublethal heat treatment when the plating medium was adjusted to pH 5.6 to 6.0. Unheated controls gave similar counts with media of pH in the range of 5 to 9.

It has been shown that the growth of heat-treated Staph. aureus (Stiles and Witter, 1965) and Strep. faecalis (Clark, Witter and Ordal, 1968) is inhibited in selective media used for these organisms, while the same cells grow normally on nonselective media. It was shown that the heating process damaged the cells to an extent that they could not grow and multiply in the presence of selective agents and yet behaved normally when not challenged by these agents. Busta and Jezeski (1963) observed decreasing counts of sublethally-heated Staph. aureus cells as the sodium chloride concentration of modified S-110 agar (Difco) was increased. Stiles and Witter (1965) calculated thermal death rates of Staph. aureus in 10% reconstituted skimmilk and showed that $D_{54.9^{\circ}\text{C}}$ values varied inversely with the amount of salt added to the growth medium. The control medium (trypticase soy agar) showed a $D_{54.9^{\circ}\text{C}}$ value of 40 min while the addition of a 7.5% (w/v) sodium chloride solution showed a $D_{54.9^{\circ}\text{C}}$ value of 6 min. Iandolo and Ordal (1966) observed that on heating Staph. aureus cells at 55°C for 5 min, 90% of the viable population failed to grow on trypticase soy agar containing sodium chloride (7.5%, w/v); up to 10 min more heating time resulted in a proportionate increase in the

number of salt-sensitive cells, whereas heating longer than 15 min induced little additional salt sensitivity.

Heat-treated bacterial cells can be distinguished from unheated cells by the parameters mentioned previously. Injured cells showing these characteristics can, under suitable conditions, recover without growth, and once recovered are indistinguishable from unheated cells. This repair of damage after heat treatment has been termed recovery in this study, and does not refer to the isolation of organisms from their environment. In this respect, Iandolo and Ordal (1965) have misinterpreted Harris (1963) who defines recovery as "'getting back' organisms from their (sub-lethal) environment." Harris uses the term revival to "imply the process of 'getting better'."

It has been shown that heated Staph. aureus cells recover their normal tolerance to sodium chloride (Stiles and Witter, 1965) while being incubated at 37°C in trypticase soy broth or in 10% reconstituted skimmilk. Stiles and Witter observed this recovery in 5% glucose or 5% galactose solutions and also noted that penicillin, vancomycin, chloramphenicol and EDTA failed to inhibit the recovery process. Actinomycin D at 5 µg/ml interfered with the recovery of salt tolerance of injured Staph. aureus cells (Iandolo and Ordal, 1966), indicating that the recovery mechanism involved RNA synthesis.

Heat-injured Strep. faecalis cells were seen to recover their tolerance to sodium chloride, sodium azide, and bromcresol purple when incubated in tryptic soy broth (Clark, Witter and Ordal, 1968). This recovery process was slower in a complete synthetic medium.

Actinomycin D also inhibited the recovery process of Strep. faecalis and thus demonstrated the requirement for RNA synthesis in the recovery mechanism.

The increased sensitivity to selective agents after heating is of prime importance to the applied bacteriologist when using selective media to enumerate microorganisms. Negative results may be false in that injured cells may be present which could recover. Heat injury and selective media for Staph. aureus have received much attention in an attempt to improve the accuracy of the detection of this organism in foods. Egg yolk has been shown to decrease the selectivity of media shown towards injured Staph. aureus cells (Clark, 1968), and on this basis egg yolk telurite glycine pyruvate agar and egg yolk azide agar were recommended for the enumeration of heat-treated Staph. aureus.

While increased sensitivity to selective media has been studied extensively for heated Gram-positive organisms, little work has been reported on this characteristic of heat-treated Gram-negative organisms such as E. coli. In testing foods for the presence of E. coli selective media must be used to distinguish them from the many other microorganisms that might be present. Public health standards require the detection of small numbers of these microorganisms, and therefore the effect of selective agents upon the growth of these organisms becomes important. Furthermore, salmonellae in foods represent a potential health hazard, therefore it is important that their detection should be accurate. The purpose of this study was to estimate the

reliability of selective media for enumerating or detecting heat-treated cells of E. coli and Salmonella typhimurium.

MATERIALS AND METHODS

Cultures

E. coli strains SA603 (E. coli K) and SA211 (E. coli B) (obtained from Dr. K. Sanderson, Department of Biology, University of Calgary, Calgary, Alberta) were selected for their streptomycin resistance. Streptomycin resistant strains were required to allow identification of the test strain in cheese-making trials. Salmonella typhimurium strain 13311 from the American Type Culture Collection was used as the test organism for the salmonella studies.

Propagation

The cultures were transferred daily into 100-ml sterile tryptic soy broth (Difco) and incubated in a New Brunswick Gyrotory shaking incubator at 37°C for 24 hr. The cultures were checked daily for purity by microscopic examination of a Gram-stained smear. Periodic checks were done on the E. coli cultures to check their resistance to 100 µg/ml of streptomycin sulfate. The 16 to 24 hr daily cultures were used to inoculate 200 ml sterile tryptic soy broth to OD_{600 mµ} of 0.1 for incubation at 37°C to be used in the individual experiments. (All optical density measurements were done in a Bausch and Lomb Spectronic 20 spectrophotometer at 600 mµ.) Under these subculturing conditions, the cultures grew with minimal or no lag period to OD 1.0 within 2 1/2 to 3 hr.

Preparation

The cultures grown to $OD\ 1.0 \pm 0.04$ were harvested by centrifugation in a refrigerated centrifuge (Sorvall RC-2B, GSA head) at 5,000 r.p.m. for 5 min. The supernatant was decanted and the cells resuspended in sterile 0.85% (w/v) saline solution.

Heat treatment

Sterile tryptic soy broth was used as the heating menstruum. A 99 ml volume of the sterile broth, in a 500-ml Pyrex Erlenmeyer flask, was preheated to the desired heating temperature. The heating system was a modification of that used by El-Bisi and Ordal (1956), using the Erlenmeyer flask as the reaction chamber and a Colora constant temperature recirculating water bath as the primary heating chamber. The broth was stirred by means of a magnetic stirrer and 1 1/2-inch Teflon covered stirring bar during preheating and the heating period. A 1 ml aliquot of the resuspended cells of the test organism was added to the preheated heating menstruum by pipet. A period of 30 sec was allowed for temperature equilibration and mixing in the reaction chamber, and the time zero sample was withdrawn 30 sec after inoculation.

Sampling

Successive 1 ml aliquots of the heated cell suspension were removed by pipet at sampling times of 0, 5, 10 and 15 min. These samples were transferred into 9 or 99 ml precooled (0°C, in an ice bath) sterile 0.1% (w/v) peptone water blanks, in preparation for

further dilution and plating. In some cases, exceptions to this procedure were necessary; for example, where the expected concentration of the viable organisms/ml was such that 0.1 or 1.0 ml aliquots of the heated cells were plated.

Recovery

At the end of the heating period, the reaction flask was removed from the secondary heating chamber, and cooled to the recovery temperature in the ice bath. Recovery was studied at 4.4°C in standing cultures, and at 20° and 37°C in shaken cultures. Sampling at 4.4°C was done at 12 to 24 hr intervals, and at 20° and 37°C at 30 or 60 min intervals. Samples were inoculated into cooled 0.1% peptone water blanks, and an aliquot was used to determine the optical density. Sampling was continued until an increase in the optical density was observed.

Plating

The samples were stored in the ice bath until they could be plated. Immediately prior to plating, the samples were diluted to the required level and appropriate aliquots were inoculated into the sterile plastic petri dishes or test tubes containing broth.

For the E. coli studies, the dilutions were inoculated in triplicate for solid media, and in quintuplicate for the most probable number (MPN) determinations in the broths. The solid media used in these studies were all from Difco and were: nutrient agar (control), violet red bile agar, MacConkey agar, desoxycholate agar,

desoxycholate lactose agar and eosin methylene blue agar. To determine the MPN of E. coli, the liquid media (Difco) were: nutrient broth (control), lactose broth, BDG broth, brilliant green bile 2% broth, E C medium broth, formate ricinoleate broth, MacConkey broth and lauryl tryptose broth. In the MPN studies involving liquid media nutrient agar and violet red bile agar were used as selective and nonselective solid medium controls, for reference purposes.

For the salmonellae studies, the solid media used for the pour plate technique were: nutrient agar (control), Endo agar and MacConkey agar. Media used for the surface plate technique were: nutrient agar (control), Endo agar, MacConkey agar and brilliant green MacConkey agar. All the media used in the salmonellae studies were supplied by Difco with the exception of brilliant green MacConkey agar which was prepared following the recommendations of Thatcher and Clark (1968). For pour plates, platings were carried out in triplicate; but for prepoured plating, in which 0.1 ml was inoculated on the surface of a 24-hr prepoured plate, the platings were carried out in quadruplicate for each dilution. The inoculum was spread with an alcohol-flamed glass "hockey stick", and the first plate discarded. This is because the first plate in the series of surface streaked plates gives a count significantly different from that of the subsequent plates (Stiles, unpublished data). In both cases, the mean counts were based on the counts from three plates. The enrichment broths for salmonellae were inoculated in quintuplicate to determine the MPN. Some of the enrichment broths inhibited the growth of cells to such

an extent that it was not possible to determine the MPN since all dilutions often failed to exhibit growth. It was therefore decided to inoculate in triplicate 0.1-ml aliquots of serial dilutions of heated (10 min at 52°C) and unheated cultures into the enrichment broths shown in Table 8 to determine the inhibitory action of the enrichment broths. It is recommended for some of these media that the tubes be filled to a depth of 2 inches (Difco Laboratories, 1953) and for these, larger volumes were used and are noted in Table 8.

Incubation

The inoculated plates or tubes were incubated at 37°C for 24 $\pm$ 3 hr. However, the colonies of the heat-treated E. coli samples grown on nutrient and other nonselective media were too small to count after 24 hr, and they were incubated for a further 12 to 24 hr. Similarly, the broth cultures for E. coli were examined after 24 hr, and re-incubated at 37°C for a further 24 hr. In view of the considerable number of tubes inoculated with heated cells that showed no growth at 24 hr but growth after 48 hr, some trials were extended for a further 24 hr to determine if further growth occurred after 72 hr. In all cases, 48 hr incubation was sufficient for the growth of the inoculated tubes. For the salmonellae broth studies, growth of the salmonellae could not be determined visually in any broths except nutrient and cooked meat broths. To check for growth, the selective enrichment broths were streaked on pre-poured nutrient agar plates

Table 8 Enrichment broths used for
Salmonella typhimurium injury studies

Medium	Brand	Lot Number	Volume per tube (ml)
Cooked meat medium	Difco	468914	9
Nutrient broth	Difco	529539	9
Tetrathionate broth	Difco	510550	9
Tetrathionate broth	Fisher	743899	9
Mannitol selenite broth	Oxoid	4952	15
Selenite broth	Difco	464134	15
Selenite broth	Difco	496860	15
Selenite cysteine broth	Difco	525623	15
T T broth	Difco	514572	9
Selenite F broth	BBL	3713	15
S B G broth	Difco	525529	9
S B G-sulfa broth	Difco	527805	9

after 24 (and 48) hr incubation at 37°C. The nutrient agar plates were incubated at 37°C for 24 hr and examined for growth.

Chloramphenicol studies

In the studies where chloramphenicol was added to the tryptic soy broth growth and recovery media, 2.5 µg/ml chloramphenicol (Parke, Davis and Co.) was added. All other conditions of the experiments were kept the same.

Bile salts studies

To determine the extent bile salts would inhibit the growth and multiplication of injured E. coli cells, Bile Salts No. 3 (Difco) were added to nutrient agar at a concentration of 0.15 g/liter. Also violet red bile agar was prepared which was devoid of Bile Salts No. 3. These media plus nutrient agar and violet red bile agar, nonselective and selective controls, were used in heating trials.

Cottage cheese simulation studies

The purpose was to simulate conditions that might exist in cottage cheese curd with respect to pH and chemical environment rather than to carry out tests on cottage cheese curd per se. To do this, skimmilk powder was reconstituted to 11% (w/v) total solids, and the pH adjusted to 5.0 and 6.5 with 1 N HCl after sterilization. This was used as the heating menstruum for these experiments.

Calculations

Where possible, D values for the specific temperature and heating conditions were calculated. These data were obtained from the reciprocal of the best fit straight line, calculated using a simple regression program in an APL/360 computer system (Smillie, 1969).

VERIFICATION OF METHODS

Streptomycin tolerance of the *E. coli* strains

To determine the streptomycin tolerance of the *E. coli* test strains, streptomycin sulfate (Pfizer) powder was added to plate count agar (Difco) in concentrations up to 1,000 $\mu\text{g/ml}$. The streptomycin sulfate powder was dissolved in distilled water and sterilized by filtration (Millipore bacterial filter, 0.45 μ pore size). The streptomycin solution was added aseptically to the sterile plate count agar at 45°C, immediately before use. The test organisms in the stationary growth phase [1% (v/v) inoculum in tryptic soy broth, in shake culture at 37°C for 8 hr] were plated in quintuplicate on plate count agar containing different concentrations of streptomycin. The plates were incubated at 37°C for 30 hr before counting.

The results indicated that both *E. coli* strains were slightly sensitive to streptomycin sulfate present at a concentration of 200 $\mu\text{g/ml}$. At 100 $\mu\text{g/ml}$ the count was not affected, and this concentration was selected for further studies. Wild type *E. coli* were shown to be sensitive to concentrations of 10 $\mu\text{g/ml}$.

Effect of storage in diluents on viability of *E. coli*

To determine the effect of holding the *E. coli* cells in diluents at 0°C and 20°C, serial dilutions of heated (55°C for 30 sec) and unheated cells were prepared in 1/4 strength sterile Ringer's solution (Oxoid) and 0.1% (w/v) sterile peptone solution. The four samples were plated at half-hour intervals for 3 hr, on plate count agar,

plate count agar with 100 µg/ml streptomycin sulfate, nutrient agar, and violet red bile agar. The least change in the count was noted in the 0.1% peptone dilutions stored at 0°C. Since it was anticipated that storage times of up to 1 hr might occur during the heating experiments, 0.1% peptone water stored on ice (0°C) was used as the diluent throughout these studies.

Control media

To determine the efficiency of the selective media for the enumeration of the E. coli strains or salmonellae, a reference control, representing 100% growth, was necessary. Ideally, the control medium would show the slowest death rate of all the media tested, and would not increase or decrease the count during the recovery period. In the recovery studies by Stiles and Witter (1965) and Clark (1968), it was noted that with Staph. aureus there was a slight decrease in count on tryptic soy agar during the recovery period. For the E. coli strains the Difco media examined as potential controls were: nutrient agar, plate count agar, plate count agar plus 100 µg/ml streptomycin sulfate, tryptic soy agar, tryptic soy agar plus 1 ml/100 ml egg yolk (Oxoid), and tryptone glucose extract agar. For Salm. typhimurium, four media (Difco) were examined as potential controls: nutrient agar, tryptic soy agar, tryptone glucose extract agar, and plate count agar.

Cultures of E. coli and Salm. typhimurium were heated at 52°C for 10 min and allowed to recover at 37°C. Unheated, heated and recovery samples were plated on the media cited above and incubated at 37°C

for 24 ± 3 hr. Salmonellae samples were enumerated by the pour plate and surface plate technique.

Contrary to the results obtained with Staph. aureus, all of these potential control media showed some increase in numbers during the early stages of the recovery period after heat treatment. After this initial increase, the counts levelled off to a lag phase, prior to commencing the growth phase. Studies on the nature of this initial increase in number (whether recovery or growth) are shown and discussed in the text (p. 75). However, nutrient agar gave the highest counts and was therefore selected as the control medium throughout these studies. This selection could not be made without reservation because of the change in numbers at the start of recovery, especially with E. coli strain SA211 (see Figure 9 , p. 74).

Selective media for Salm. typhimurium

Media were evaluated for their potential use as enumeration media for salmonellae. A culture grown to OD 1.02 was plated in triplicate using both pour and surface plating techniques on the following media: bismuth sulfite agar, brilliant green MacConkey agar, DCLS agar, desoxycholate citrate agar, Endo agar, eosine methylene blue agar, MacConkey agar, brilliant green agar, S S agar, and brilliant green-sulfa agar. Nutrient agar was used as a control. Media other than those listed on page 37 were rejected due to the low counts compared to nutrient agar and/or the difficulty of observing colonies because of the opacity of the medium. However,

brilliant green MacConkey agar was incorporated in the studies, even though it gave low counts compared to nutrient agar. This exception was made because brilliant green MacConkey agar was recently recommended for use in salmonellae detection by Thatcher and Clark (1968).

RESULTS AND DISCUSSION

Heat injury of *E. coli* as shown by solid media

Pure cultures of *E. coli* SA603 heated in tryptic soy broth (Difco) were plated on the various selective and control (nonselective) media listed on page 36. The data from the enumeration of the survivors on these media were used to prepare thermal death curves. The survivor data were subjected to a simple regression analysis. The reciprocal of the slope of this thermal death curve is, by definition, the D value. This is the time required to destroy 90% of the population. As the heating conditions for the cells in any comparison were identical, the death rates or D values reflected on the different media should be the same, unless the media were exhibiting a further inhibitory or lethal effect on the survivors. This phenomenon has been referred to as "heat injury" and is discussed in the introduction.

The mean $D_{52^{\circ}\text{C}}$ values for *E. coli* SA603 are given in Table 9. The number of observations made on each medium differed. Nutrient agar was used as the nonselective control medium in all experiments. Violet red bile agar is the most widely used selective solid medium for the detection and enumeration of coliforms. Therefore, nutrient agar and violet red bile agar were used in most of the trials as non-inhibitory and inhibitory controls, respectively. Eosin methylene blue agar was used in only two trials, as colonies in this medium were too difficult to count due to the dark color of the medium. As this medium could not be used for the enumeration of *E. coli* it was

Table 9 Mean $D_{52^{\circ}\text{C}}$ values for *E. coli* SA603

Medium*	$D_{52^{\circ}\text{C}}$ (min)	Trials
Nutrient agar	21.06	9
Violet red bile agar	3.91	12
Desoxycholate agar	5.57	4
MacConkey agar (100°C/15 min)	5.73	2
MacConkey agar (121°C/15 min)	5.86	2
Desoxycholate lactose agar (100°C/15 min)	6.23	4
Desoxycholate lactose agar (100°C/15 min)	6.22	4
Eosin methylene blue agar (100°C/15 min)	4.78	2
Eosin methylene blue agar (121°C/15 min)	4.73	2

*Sterilized according to Difco instructions. Parentheses indicate method used where alternatives are suggested.

eliminated from further studies. MacConkey agar was used in a further two trials. However, extremely unreliable counts, apparently due to inaccuracies in the serial dilutions, were obtained, and these results were rejected. The data from two subsequent trials revealed similar inaccuracies. This was shown not to be due to dilution errors, and the apparent cause of this inaccuracy will be discussed.

The $D_{52^{\circ}\text{C}}$ values in Table 9 indicate that the different media affect the apparent death rate of the organism (heat injury). It is important to note the large differences between mean $D_{52^{\circ}\text{C}}$ values for nutrient agar (21.06 min) and those for the selective media, which range from one third to one fifth of the values observed on nutrient agar. Large differences were observed between replicates. Possible reasons for these differences will be discussed later. The object of this study was not to determine precise D values, but rather to compare the D_{nutrient} value against the $D_{\text{selective media}}$ values. In all trials, the $D_{52^{\circ}\text{C}}$ values obtained using the selective media were markedly less than the $D_{52^{\circ}\text{C}}$ values obtained on nutrient agar.

The death curves of SA603 obtained on nutrient agar and violet red bile agar were linear, indicating a proportional increase in injury and death with increased time of heating. After 15 min heating at 52°C the mean percentage injury as shown on violet red bile agar was 99.97% (Table 10).

The Difco manual recommends that MacConkey agar, desoxycholate lactose agar, and eosin methylene blue agar be used when "heated only to boiling to dissolve it completely before pouring" or, if it

Table 10 The reduction of counts of *E. coli* SA603
on violet red bile agar as compared with nutrient agar (8 trials)

Heating time at 52°C	Mean percentage reduction
Before heating	24.5
0 min	86.9
5 min	98.87
10 min	99.93
15 min	99.97

is to be stored more than 24 hr, sterilized in the "autoclave for 15 min at 15 pounds pressure." Hence these media were used in two trials, boiled and autoclaved, to test for differences due to sterilizing treatment. As is seen in Table 9 the differences are insignificant.

As noted earlier the $D_{52^{\circ}\text{C}}$ values often showed considerable differences between trials. The mean $D_{52^{\circ}\text{C}}$ value for strain SA603 obtained from nine trials for nutrient agar was 21.06 ± 9.12 min. Similarly, violet red bile agar in 12 trials resulted in a mean $D_{52^{\circ}\text{C}}$ value of 3.91 ± 1.41 min. The large variations reflected by the standard error of the means could perhaps be caused by such factors as differences in the individual cells, and time of sample storage in diluent, in addition to normal experimental error. Although every effort was made to keep these inherent errors to a minimum, the possible influence of sample storage time can be appreciated when it is realized that four samples were taken in the 15 min heating period. From these samples, three dilutions were plated in triplicate on eight media. In other words, it was necessary to inoculate and pour approximately 300 plates. This was generally achieved within 30 to 60 min of the start of the experiment.

When the large variations in D values were realized, two thermal death time trials were run on the same day to establish the variability between trials performed on one day. It was felt that this would keep variations in cell behavior to a minimum. The flasks of tryptic soy broth were inoculated 30 min apart from the same

mother culture, grown to OD 1.0 then heated and plated. The $D_{52^{\circ}\text{C}}$ values for E. coli SA603 on nutrient agar from all nine trials ranged from 8.9 to 38.46 min, with a mean of 21.06 min, while the $D_{52^{\circ}\text{C}}$ values obtained on the replicate trials were 25.64 and 20.83 min. Similarly $D_{52^{\circ}\text{C}}$ values for violet red bile agar from all 12 trials ranged from 2.50 to 7.69 min with a mean of 3.9 min. The $D_{52^{\circ}\text{C}}$ values of the replicates obtained on the same day were 3.92 and 3.62 min. Although these replicate $D_{52^{\circ}\text{C}}$ values obtained on the same day appear relatively close it could not be stated conclusively that studies carried out on one day were more likely to give closer results than studies on different days. This closeness could have been due to chance and a more extensive study would be required for confirmation. It does seem probable that the variation could be reduced by determining replicate D values within a limited time interval. However, the number of trials that could be carried out on one day was limited by the technical operations required to obtain the results. Additional limitations applied to the number of trials that could be carried out on successive days. Again it is emphasized that the determination of precise D values was considered secondary to subjecting the organisms of one trial to all of the media in question at the same time. Otherwise the comparisons between media would not have been valid.

A further possible reason for variation of results was noted in the work with strain SA603. As shown in Figure 3, a 1/10 dilution of heated cells did not result in a proportionate reduction

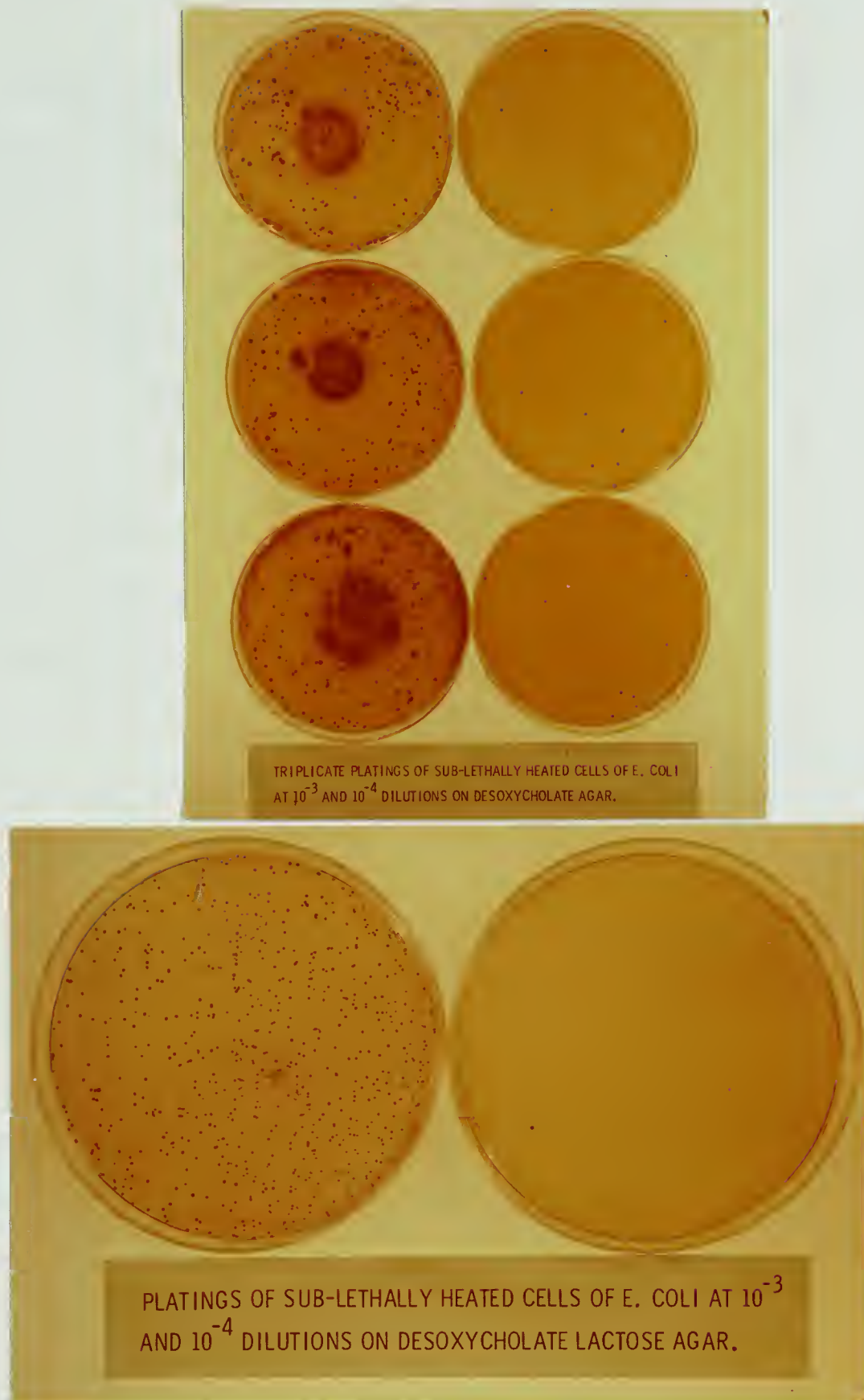


Figure 3. Differences in colony growth of E. coli SA603 when plated with a 10-fold dilution difference on selective media after sublethal heat treatment.

in colony numbers on selective media. The 10^{-4} dilution of heat-injured strain SA603 resulted in less than 30 colonies/plate, while the 10^{-3} dilution gave concentrations of colonies too numerous to count. However, reliable serial dilutions were observed on the control medium. The possibility of this phenomenon being caused by a dilution error was checked in further experiments and the same results were observed. To investigate this phenomenon, intermediate dilutions were made of unheated cells and cells taken from a culture one half hour after heating at 52°C for 15 min. The following protocol shows the dilutions used:

	Intermediate dilutions					
	0.1	0.2	0.4	0.6	0.8	1.0
Volume of appropriate dilution of cell suspension	1	2	4	6	8	10
Volume of sterile 0.1% diluent solution	9	8	6	4	2	0

A 0.1 ml aliquot of each dilution was plated in triplicate into nutrient and violet red bile agar and other selective media. On an arithmetic scale, linearity would be expected when the cell count was plotted against dilution. The results for unheated cells revealed the expected relationship on both the selective and control media. Increasing the dilution by a factor of 10 resulted in the expected decrease of approximately 10 times the count. The heated cells gave comparable linear results when plated on nutrient agar, the curve

being similar to the theoretical curve (Figure 4). Selective media such as violet red bile agar gave results which deviated markedly from the expected 10-fold relationship. The data plotted in Figure 4 were presented as a semilogarithmic plot in order to illustrate the 100-fold increase in the count that occurred with a simple doubling of the concentration. From this, it appeared that the organisms must be present in a concentration of over 300/plate on the selective media before they are able to overcome this inhibitory effect. It has recently been shown that recovering Staph. aureus cells are metabolically active and while the cells are not able to multiply during the recovery process they still produce some end products (Bluhm and Ordal, 1969). If the heat-injured *E. coli* are metabolically active, in large concentration they may be able to alter the surrounding medium. In the case of selective media containing bile salts, it might be the precipitation or removal of the bile salts by these metabolically active cells that enabled the injured cells to multiply. Further studies are required on this phenomenon before valid conclusions can be drawn.

For *E. coli* SA211 it appeared that these large populations were not necessary for proportional growth on the selective solid media. On heating this strain at 52°C, apparent linear death curves were obtained on nutrient agar, while the death curves obtained on violet red bile agar and other selective media were nonlinear during the same period of heating.

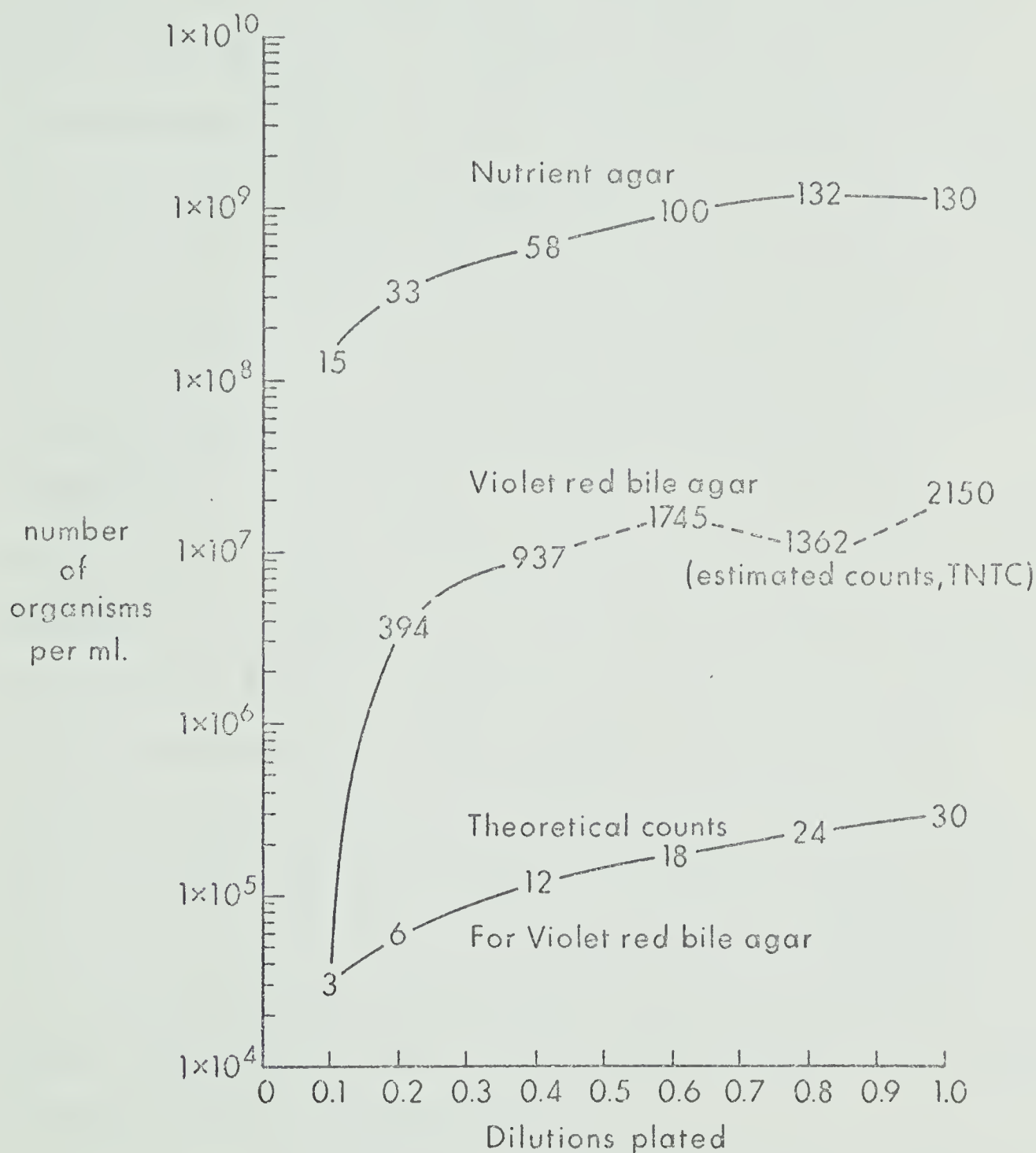


Figure 4. The effect of dilution on the counts of heated cells of *E. coli* on selective and nonselective media.

(Numbers on curves represent actual number of colonies on plates.)

To examine these nonlinear data obtained on violet red bile agar, the mean slopes of the death curves over the successive 5 min intervals (0 to 5, 5 to 10, 10 to 15 min) were calculated from seven trials (Figure 5). As the slope of this death curve is probably decreasing continuously, it would more correctly be represented by a smooth curve than this discontinuous curve. The death curve on nutrient agar was linear during the first 15 min of heating at 52°C so a mean $D_{52^{\circ}\text{C}}$ value for the overall heating period could be calculated for the seven trials. The death curves obtained when the heated cells of strain SA211 were plated on MacConkey, desoxycholate and desoxycholate lactose agars were similar to the curve shown for violet red bile agar in Figure 5. Hence these results will not be considered separately. The nonlinearity of the death curve observed on violet red bile agar resulted in differences in levels of injury during the heating period for strain SA211, compared to the proportional increase in injury observed with strain SA603. With strain SA211, the part of the death curve determined on nutrient agar was linear, while that on violet red bile agar was asymptotic. This indicated that during the 15 min heating period, the level of injury was at a maximum (98.9%) after 5 min of heating, but decreased as the heating continued (97.0% at 15 min)(Table 11).

Mean counts of unheated E. coli SA211 obtained on violet red bile agar were 33.2% lower than those obtained on nutrient agar (Table 11). Similarly, unheated E. coli SA603 gave counts on violet red bile agar which were 24.5 % lower than those of nutrient

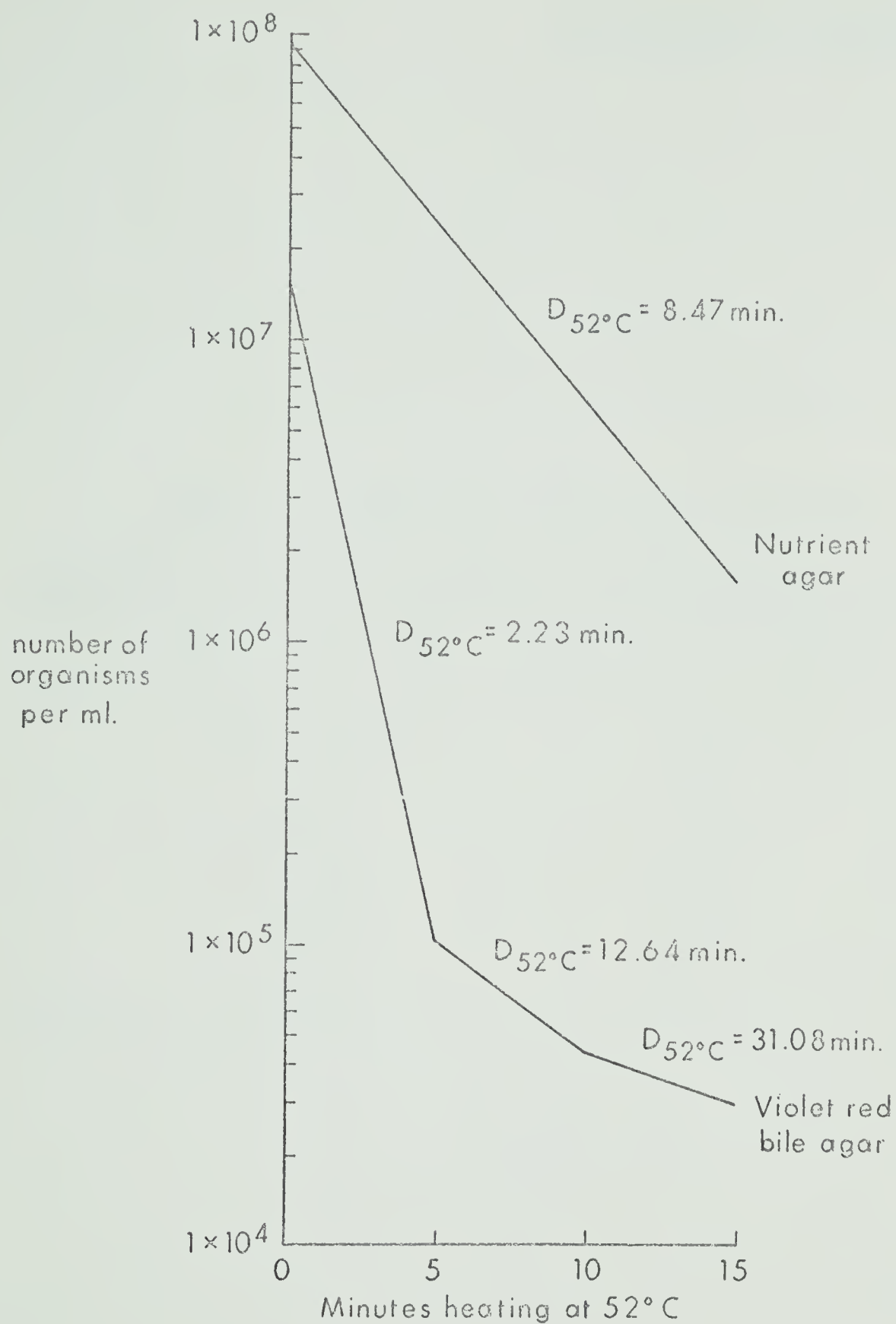


Figure 5. Mean thermal death curves for E. coli SA211 from seven trials as determined on nutrient agar and violet red bile agar.

Table 11 Reduction of counts of *E. coli* SA211
on violet red bile agar as compared with nutrient agar (7 trials)

Heating time at 52°C	Mean percentage reduction
Before heating	33.2
0 min	82.8
5 min	98.9
10 min	98.03
15 min	97.0

agar (Table 10). These differences are not uncommon and are probably due to the selectivity of the medium. It is interesting that neither Standard Methods (1965) nor Thatcher and Clark (1968) in their text on food microbiology techniques warned their readers of such differences, although as early as 1935 Wilson reported that the count of "Bacterium coli" obtained on MacConkey agar was 25% lower than that on "heart extract" agar.

Yet another effect of heat injury must be recorded. It was observed in these studies that after 24 hr of incubation, colonies from heated cultures growing on nonselective (control) media were generally smaller than colonies from unheated cultures. This made the nutrient agar plates from the heated cultures difficult to count at 24 hr, and they had to be incubated for a further 12 to 24 hr before they were enumerated. A similar observation was noted by Strange and Shon (1964) for heat-treated cultures of Aerobacter aerogenes on T M agar (Douglas' Digest). This small colony size can be explained from Jackson and Woodbine's (1963) observation that sublethally-heated cells of Staph. aureus exhibited an extended lag phase. This phenomenon has also been shown for E. coli, and is reported in the recovery section of this thesis. As a result of this extended lag phase longer incubation times would be expected for heated cells.

Conversely, unheated colonies growing for 24 hr on selective media were not difficult to count. This was due to the fact that the typical rose coloration of the colonies and the surrounding

medium, made the colonies on the selective media easy to identify after 24 hr incubation. Counts obtained after 48 hr incubation on the selective media seldom varied from those obtained after 24 hr.

Heat injury of *E. coli* as shown by liquid media

The multiple-tube fermentation technique, using the broths shown on page 37, was used to enumerate cultures of *E. coli* during a 15-min heating interval at 52°C. Most probable number (MPN) estimates show a larger experimental error than do colony counts on solid media. A generalized picture of death curves of triplicate trials after 48 hr incubation, is shown in Figure 6.

The slowest death rate for strain SA603 was shown in the nonselective broths, nutrient and lactose. Strain SA211 deviated from this expected result in that lactose broth showed a faster death rate than three of the selective broths in two of the three trials. In the remaining trial, lactose broth showed a faster death rate than the two selective broths, BDG and lauryl tryptose. As lactose broth is commonly used in the presumptive test for coliforms, the heat injury observed with SA211 indicated the possibility of "false negatives" occurring after heat treatment.

Of the selective media, BDG (Buffered Desoxycholate Glucose) broth showed the lowest level of injury compared to nutrient broth for both strains of *E. coli*. Even though BDG broth contains 0.1 g/liter sodium desoxycholate (a purified bile salt), Hajna and Damon (1955) developed it as a presumptive medium for coliform detection.

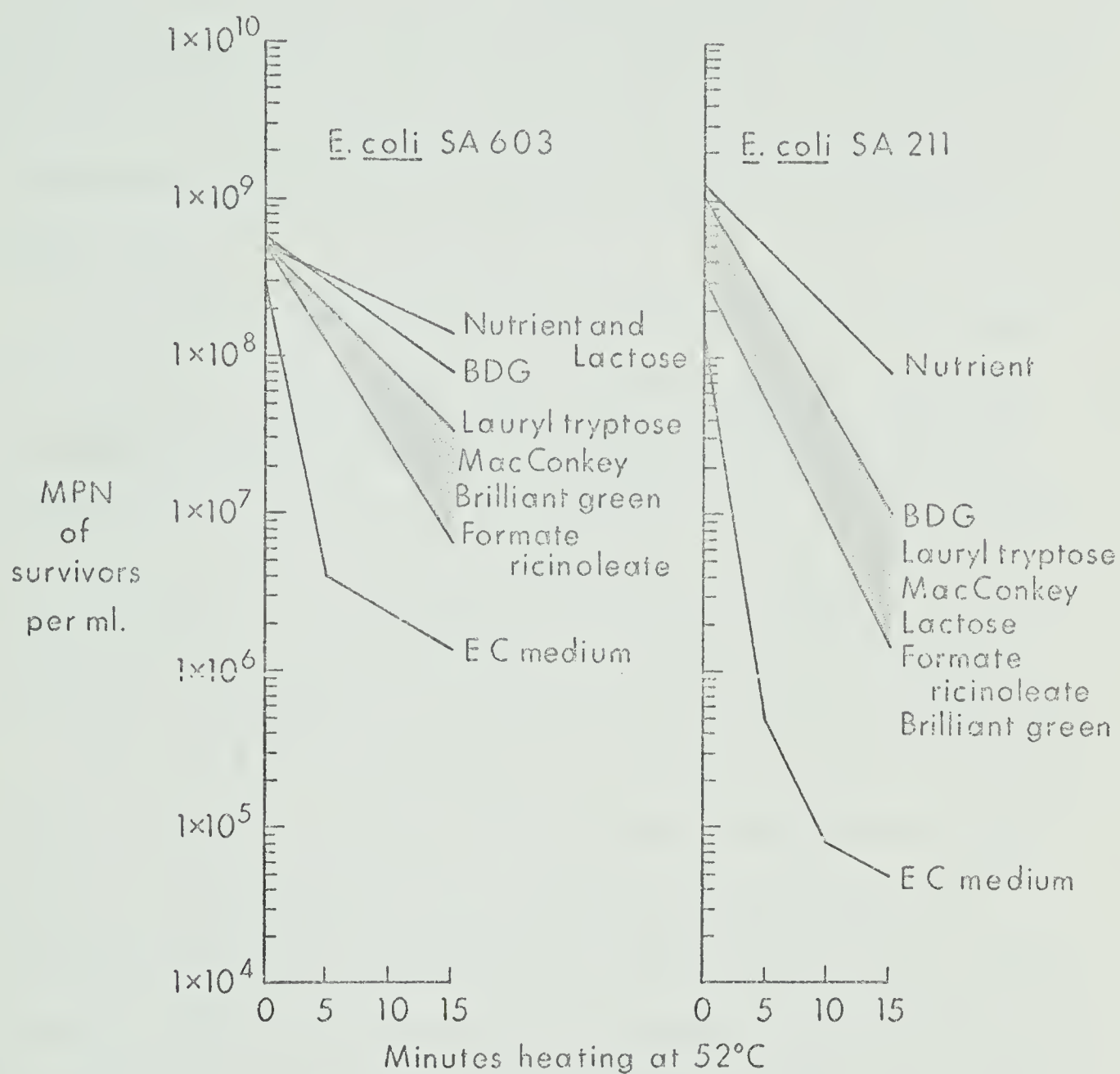


Figure 6. A comparison of generalized thermal death curves in selective and nonselective liquid media for two strains of *E. coli* heated at 52°C.

According to Hajna and Damon, it has the advantage that even the coliforms that are slow lactose fermenters produce gas during short incubation times, reducing the incidence of "false negatives." From this study it appeared that BDG broth might be considered as a medium for the presumptive test for coliforms, in preference to lactose broth.

E C medium is widely used for the identification of E. coli in the elevated temperature tests. In these studies, incubating the survivors from sublethal heat treatment at 37°C, the results obtained with E C medium were unsatisfactory. E C medium revealed the greatest amount of injury for both strains (Figure 6). The further study of the reliability of E C medium to detect E. coli at elevated temperatures (44.5°C) after heat treatment is suggested.

For all of the broths examined, with the exception of formate ricinoleate broth, unheated cell suspensions showed little change in the MPN between 24 and 48 hr incubation periods (Table 12). Standard Methods for the Examination of Water and Wastewater (1965) recommended that fermentation tubes be examined for coliform growth after 24 hr incubation, and that if no gas had been formed tubes should be reincubated for another 24 hr. Only after 48 ± 3 hr incubation was lack of gas accepted as a negative result. Standard Methods for the Examination of Dairy Products (1967) recommended that tubes be incubated for 48 ± 3 hr before being examined for coliform gas production. This requirement for 48 hr incubation became more significant when the difference in MPN between 24 and 48 hr

Table 12 The effect of increasing incubation time on the
MPN values for two strains of *E. coli* (unheated).

Broth	SA603		SA211	
	24 hr incubation at 37°C	48 hr incubation at 37°C	24 hr incubation at 37°C	48 hr incubation at 37°C
	(MPN of organisms /ml x 10 ¹⁰)			
Nutrient	4.9	4.9	7.9	7.9
Lactose	2.2	2.2	3.3	4.9
BDG	4.9	4.9	2.3	3.3
Brilliant Green Bile 2%	2.4	3.3	3.3	3.3
E C Medium	1.7	1.7	3.1	3.1
Formate Ricinoleate	0.04	1.7	2.8	14.0
Lauryl Tryptose	7.0	7.0	24.0	24.0
MacConkey	3.1	4.6	2.8	2.8

incubation was noted (Tables 13 and 14). This difference with extended incubation was seen to increase proportionally with heating time, but the extent of the discrepancy varied between media. MPN estimates with all media tested were stable after 48 hr incubation.

The nonselective broths, nutrient and lactose, and the selective broths, E C medium, lauryl tryptose, and BDG broth, showed smaller increases in MPN on extended incubation than brilliant green bile, MacConkey and formate ricinoleate broths. The E C medium which showed the most injury for both strains demonstrated little increase in count for a further incubation period (Table 14). Perry and Hajna (1944) evaluated E C medium for the isolation of coliforms and E. coli and stated that in routine work an unconfirmed presumptive test with this broth was probably more reliable than the standard completed test. The similarity between 24 and 48 hr counts tended to justify Perry and Hajna's recommendation. However, due to the large heat injury shown in this broth the results obtained may be inaccurate.

Of the media examined, formate ricinoleate broth showed the greatest difference between 24 and 48 hr incubation. The broth contains 5 g/liter of sodium formate and was designed to accelerate gas production of coliforms. The Difco manual (1953) stated:

"gas production appears earlier in this medium than in any other media under the same conditions, and that it is present in larger proportions at the completion of the test."

Because of this, formate ricinoleate would be expected to give relatively accurate results in 24 hr. However, from this study, it appeared to be the most unsuitable medium for quantitative

Table 13

The effect of incubation time on the reliability of MPN values

of E. coli for different heating times at 52°C

Strain	Heating Time (min)	Lactose		BDG		Brilliant Green		MacConkey	
		24 hr incubation at 37°C	48 hr incubation at 37°C	24 hr incubation at 37°C	48 hr incubation at 37°C	24 hr incubation at 37°C	48 hr incubation at 37°C	24 hr incubation at 37°C	48 hr incubation at 37°C
(MPN of organisms /ml x 10 ⁷)									
SA603	Before Heating	2200.0	2200.0	4900.0	4900.0	2400.0	3300.0	3100.0	4600.0
	0	49.0	49.0	49.0	49.0	22.0	22.0	17.0	17.0
	5	49.0	79.0	22.0	54.0	24.0	35.0	2.4	17.0
	10	24.0	49.0	24.0	33.0	3.3	4.9	2.4	22.0
	15	7.0	46.0	4.9	35.0	0.33	3.5	2.4	54.0
SA211	Before Heating	3300.0	4900.0	2300.0	3300.0	3300.0	3300.0	2800.0	2800.0
	0	79.0	79.0	79.0	79.0	22.0	49.0	49.0	49.0
	5	33.0	33.0	110.0	110.0	17.0	49.0	3.1	49.0
	10	1.3	11.0	23.0	23.0	0.33	2.8	3.3	4.9
	15	0.13	3.3	3.3	13.0	0.0046	0.49	0.49	2.3

Table 14 The effect of incubation time on the MPN values
of E. coli heated for 15 min at 52°C (2 trials).

Strain	Trial	Nutrient		Formate Ricinoleate		E C Medium		Lauryl Tryptose	
		24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
		incubation at 37°C	incubation at 37°C	incubation at 37°C	incubation at 37°C	incubation at 37°C	incubation at 37°C	incubation at 37°C	incubation at 37°C
(MPN of organisms /ml x 10 ⁶)									
SA603	A	24.0	170.0	0.013	92.0	7.9	7.9	49.0	240.0
	B	24.0	79.0	0.00023	0.79	0.79	0.79	2.1	>16.0
SA211	A	22.0	130.0	0.033	7.9	3.3	4.9	7.9	70.0
	B	0.35	7.9	0.0029	0.49	0.049	0.049	0.17	0.70

estimations after 24 hr of incubation for both heated and unheated cells (Tables 12 and 14).

In six trials appropriate dilutions of the cells used in the MPN estimates were plated on nutrient agar and violet red bile agar, as nonselective and selective solid media controls, respectively. This enabled a comparison of the MPN estimates with the count on these solid media. It was observed that the greater the heat treatment, the lower the nutrient agar count as compared to the MPN of nutrient broth (Table 15). This was in agreement with the results of Hershey (1939). He showed heat treated cultures of E. coli gave nutrient agar counts which were up to 99% lower than MPN estimates from nutrient broth. Hershey found that the addition of water to the agar plates increased the count and he concluded that the lower count from nutrient agar was due to a decreased available water content. It was also interesting to compare the counts obtained on violet red bile agar with the MPN of E C medium, the broth which produced the lowest MPN (Table 15). Enumeration on violet red bile agar and the MPN of E C medium compared favorably for unheated cells of SA211. However, heat-treated cells resulted in violet red bile agar counts much lower than E C medium MPN estimates (95% after 15 min heating). Similar differences between counts from solid media and the MPN estimates were observed for both E. coli SA211 and SA603.

Brilliant green bile 2% broth and MacConkey broth deserve special attention. The former is widely recommended in North America

Table 15 Comparisons of solid media counts and
most probable number estimates in broths for *E. coli* SA211

Heating Time (min)	Nutrient Agar organisms/ml	Nutrient Broth 48 hr MPN/ml	Violet Red Bile Agar organisms/ml	E C Medium 48 hr MPN/ml
Before Heating	4.2×10^{10}	7.9×10^{10}	3.7×10^{10}	3.1×10^{10}
0	5.4×10^8	4.6×10^8	3.4×10^8	7.9×10^7
5	2.7×10^8	1.3×10^9	2.6×10^6	7.9×10^7
10	8.5×10^7	7.9×10^8	1.3×10^6	3.5×10^7
15	2.2×10^7	1.3×10^8	1.8×10^5	4.9×10^6

for use in the presumptive test for coliforms in dairy products (Standard Methods, 1967), and the completed test for coliforms in water (Standard Methods, 1965). MacConkey broth is used in Great Britain in the presumptive test for coliforms in water (Ministry of Health, 1939). Both brilliant green bile 2% broth and MacConkey broth gave MPN's of unheated E. coli cultures which compared favorably with controls such as lactose and nutrient broth (Table 12). However, this was not the case for heated cell suspensions. In addition to these broths showing high levels of injury (compared to nutrient broth), there were also considerable differences between results observed after 24 and 48 hr incubation. These results indicated the need for cautious interpretations when enumerating heat-treated coliforms in these broths.

Recovery of E. coli

The prolonged lag phase observed in this study for E. coli after heat treatment is shown by the nutrient agar curve in Figure 7. These results confirmed observations of Hershey (1939) that E. coli cultures surviving heat treatment showed lengthened periods of latency. During the 3 hr incubation period following heating, the damaged cells plated on the selective media appeared to overcome their sensitivity to the inhibitors of the selective media. The count observed on violet red bile agar increased dramatically while the count on nutrient agar remained virtually constant over the same period. As mentioned earlier, the process of repair from heat injury by the sublethally heated cells is termed recovery throughout

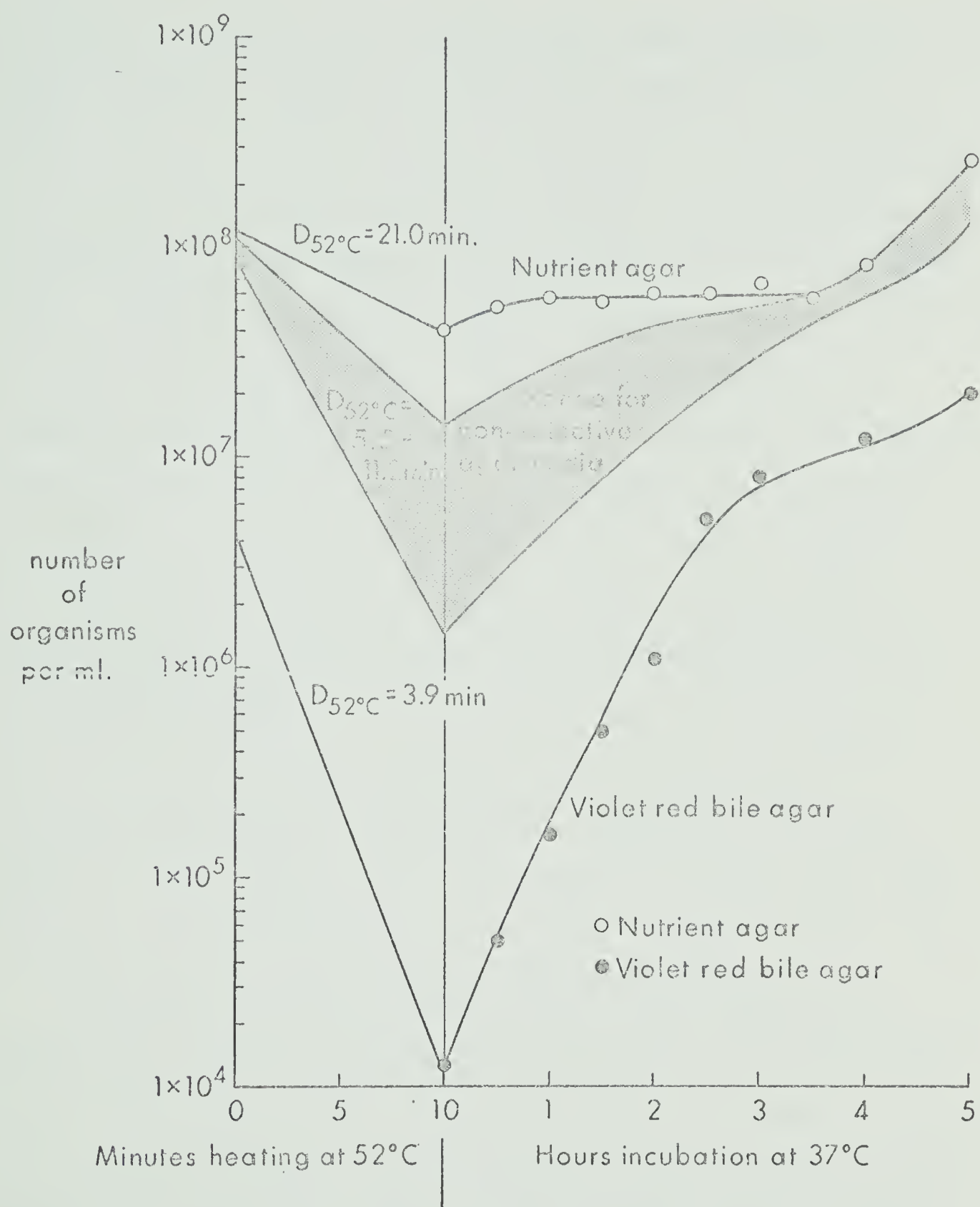


Figure 7. Mean thermal death curves and typical recovery curves for *E. coli* SA603 observed on selective and nonselective media.

this study. Recovery in this sense has been used to describe the change in ability to grow on selective media (Stiles and Witter, 1965). The curve for the recovery of E. coli SA603 shown on violet red bile agar represented the trend of the data and was generally representative of that shown for strain SA603 on the other selective media. The individual recovery curves on the other selective media for strain SA603 are not shown, since the individual points were considered unreliable due to the phenomenon of "dilution inaccuracies" observed after the heat treatment. This phenomenon was described in the section on "Injury on solid media" (p. 51). After incubation for 4 hr at 37°C in tryptic soy broth the cells of SA603 surviving the heat treatment appeared to have fully recovered their tolerance to the selective agents. The differences between counts obtained on the control and the selective media at this time were similar to differences observed for unheated cells. Furthermore, the optical density of the recovering culture only began to increase after 3 1/2 to 4 hr of recovery (Figure 8).

Although the number of viable cells of SA603 enumerated on nutrient agar increased slightly during the first 1 hr of incubation at 37°C after heat treatment (Figure 7), this was not viewed as growth, since the optical density decreased slightly during this time (Figure 8). Further, the number of cells remained constant during the subsequent 2-hr period of incubation at 37°C. The initial increase in the count shown on nutrient agar in Figure 7 was also

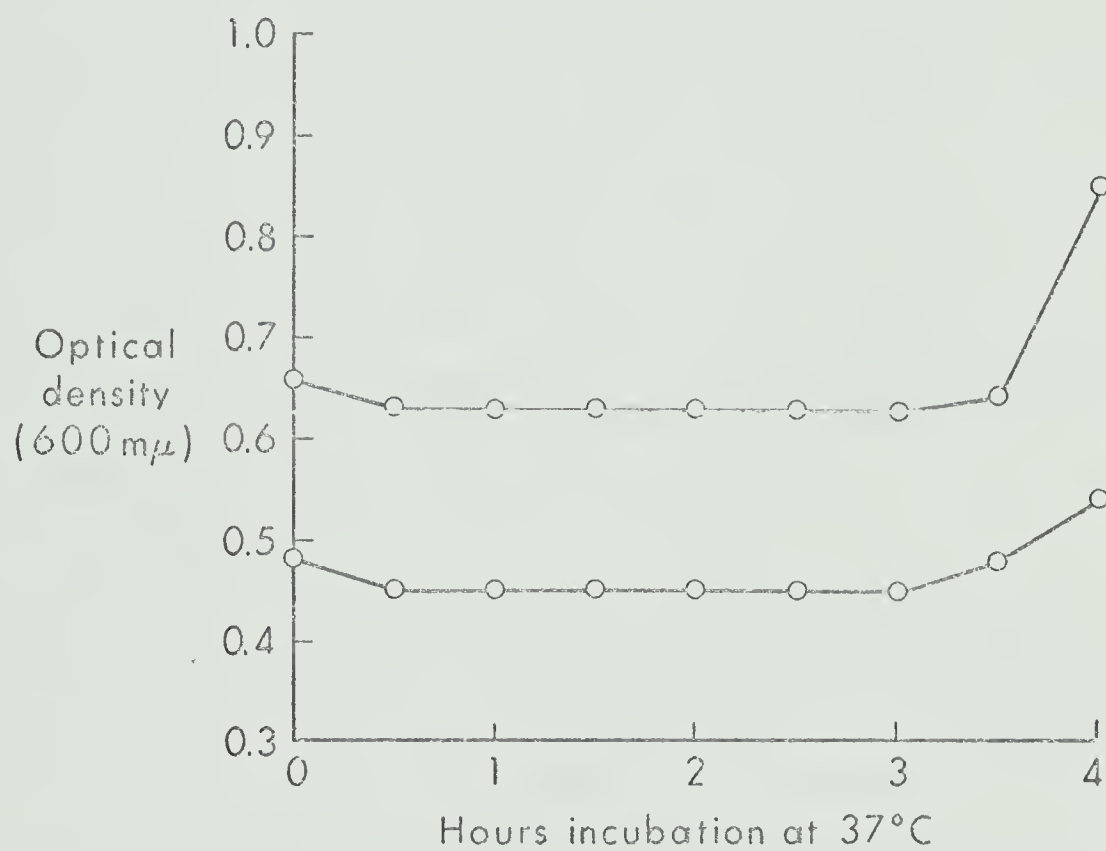


Figure 8. Optical density changes during recovery
of E. coli SA603 in tryptic soy broth,
(Results of two trials.)

shown on the other nonselective media, tryptic soy agar, plate count agar and tryptone glucose extract agar. This could not be due to a change in sensitivity to selective or inhibitory agents. However, this is probably a form of recovery in which the cells overcome their more exacting nutrient requirements that are generally observed after sublethal heat treatment (Nelson, 1943).

The recovery process for heat-injured SA603 cells was also carried out at temperatures of 20°C and 4.4°C. Recovery occurred at 20°C, but was slower than recovery at 37°C. After 4 hr of recovery at 20°C there was still a difference of 99% in the counts on nutrient agar and violet red bile agar. Recovery did not occur at 4.4°C. This was contrary to the observations with Staph. aureus (Stiles, 1963; Iandolo and Ordal, 1966). They demonstrated that recovery occurred at low temperatures (such as 4.4°C), albeit at a slower rate. Hence it appeared that the recovery process in E. coli SA603 might be inhibited by temperatures of the order of 4.4°C. More extensive work must be done on the effect of temperature on recovery.

The studies on E. coli strain SA211 revealed that this strain also recovered from the heat-induced sensitivity to the selective media. This is shown in Figure 9. The counts on the selective media during the recovery period were more reliable for this strain, because the "dilution inaccuracies" phenomenon observed with SA603 did not occur with SA211.

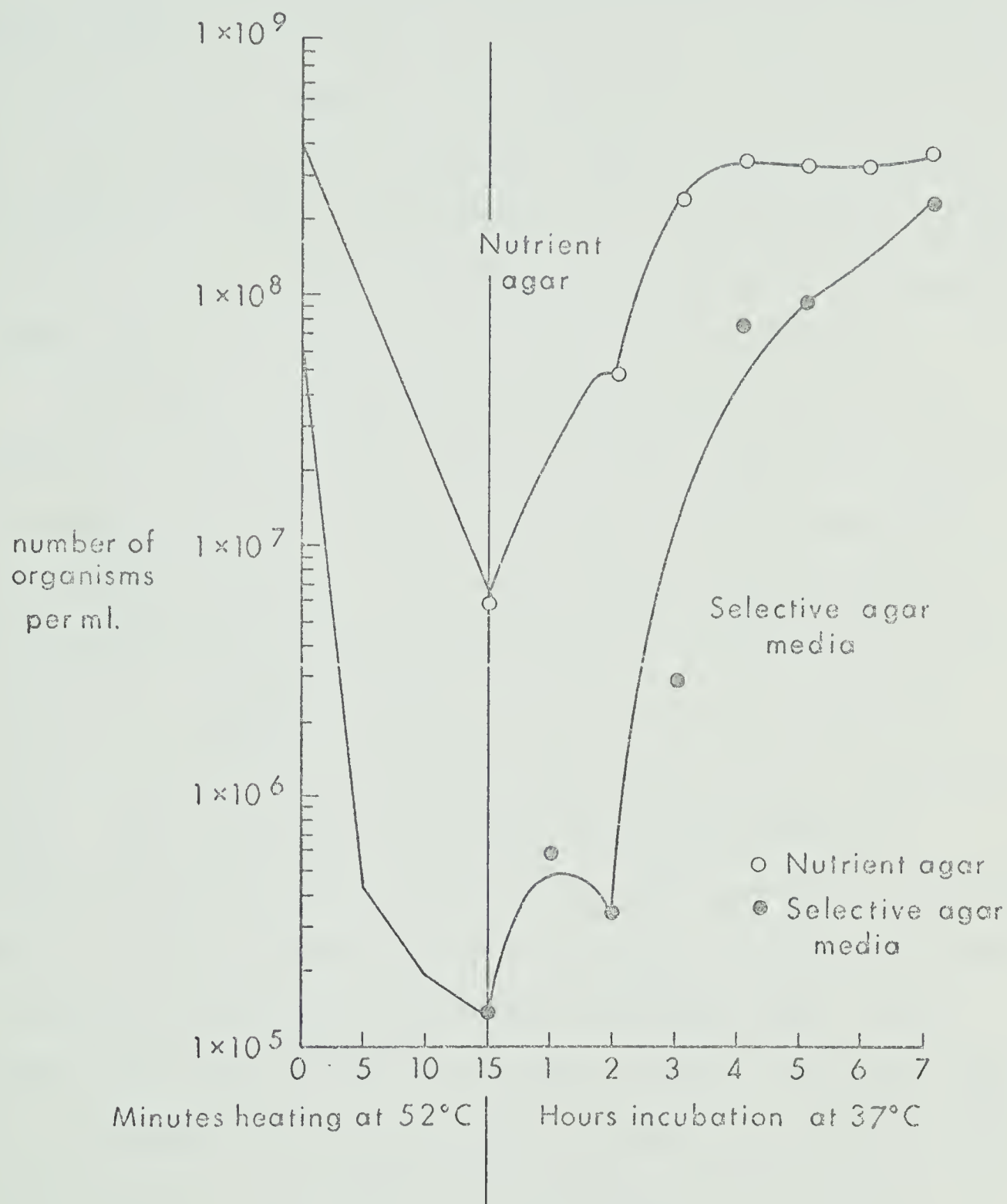


Figure 9. Mean thermal death curves and typical recovery curves for E. coli SA211 observed on selective and nonselective media.

It was noted that recovery curves for SA211, on all selective media, exhibited a sharp decrease in the recovery rates approximately 2 hr after recovery commenced. This phenomenon was apparent in all trials. As a result, the recovery curve for SA211 could not be represented by a smooth curve throughout the recovery period. However, after 7 hr, the recovery was complete and the extent of the recovery process compared favorably to that observed for strain SA603.

The length of the recovery period with SA211 was 6 to 7 hr, compared to 3 1/2 to 4 hr for SA603. This was possibly due to the 5 min extension in heat treatment given SA211, but this might also be due to strain differences. The optical density readings confirmed that no growth occurred during the first 6 hr of incubation at 37°C (Figure 10). However, the counts observed on nutrient agar with SA211 increased markedly during the early stages of the recovery period. This contrasted markedly with the limited increase in numbers during the equivalent period when SA603 was plated on nutrient agar. A similar increase occurred for E. coli cells that were damaged by ultraviolet irradiation (Hollaender and Duggan, 1938). These workers noted that this "apparent initial increase" was maximal when 94% to 98% destruction occurred and it decreased with higher or lower survival ratios. This might be an explanation for the larger increase observed for SA211 which exhibited 98.2% death while SA603 showed only 66% death during the heating period. Because of this large initial increase on nutrient agar, the exclusion of growth as a possibility in this initial period with SA211 was far more difficult

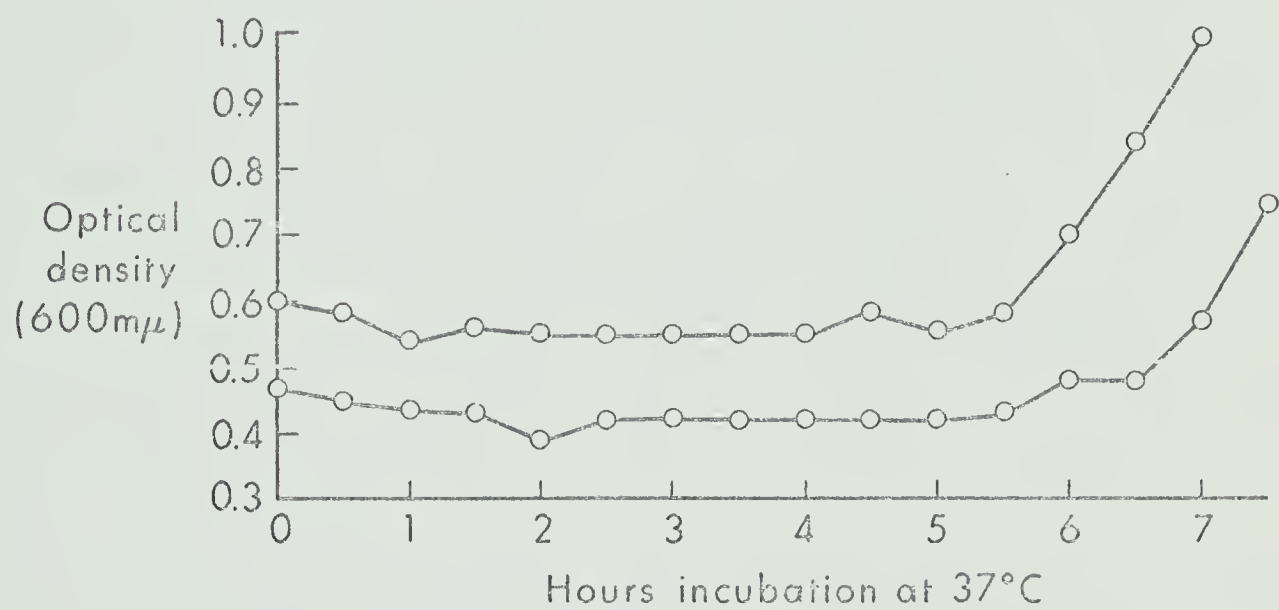


Figure 10. Optical density changes during recovery of
E. coli SA211 in tryptic soy broth.
(Results of two trials.)

to accept without question. Reference to Figure 9 indicated that although this initial "recovery" or growth represented an increase in the count on nutrient agar in the first 3 to 3 1/2 hr of incubation at 37°C, it was followed by a period of 2 1/2 to 3 hr stationary (lag) phase before typical growth commenced.

If this initial increase in cell count on nutrient agar was growth, the semi-logarithmic plot of the data should not be a curved line. In fact, these curves showed an indentation corresponding to the marked decrease in recovery rate observed on the selective medium recovery curve. Assuming this increase to be growth, and assuming linearity, the generation time would appear to be approximately 30 to 40 min. Studies on the generation times of SA211 transferred in the logarithmic phase of growth in shaking cultures at 37°C, gave generation times of 30 to 35 min. It was anticipated that the generation time for "recovery" and that for growth would be sufficiently different to exclude the possibility of growth in the former. However, the times were almost identical for the idealized (log) "recovery" and for growth. As such, "recovery" or growth was neither proved nor disproved.

In these considerations, it is important to bear in mind that during this period the optical density readings did not increase. The constant optical density readings were obtained during a period when cell numbers growing on nutrient agar changed from $6.2 \times 10^6/\text{ml}$ to $3 \times 10^8/\text{ml}$. Furthermore, if this was growth, then the data did not account for the extended lag periods reported after sublethal

heating by Hershey (1939) for E. coli, and by Jackson and Woodbine (1963) for Staph. aureus. The growth theory would also fail to account for the observation of the subsequent stationary (lag) phase after the initial increase in numbers on nutrient agar. Although the overwhelming weight of this evidence was in favor of this increase being an exaggeration of the same phenomenon recorded for SA603, it was decided to seek final proof by incorporating chloramphenicol in the recovery medium, to inhibit growth.

Prior to the recovery studies in which chloramphenicol was added to the recovery medium, a trial was carried out to determine the concentration of chloramphenicol necessary to inhibit the growth of the E. coli test strains. The results indicated that 2.5 μ g of chloramphenicol per ml of tryptic soy broth was sufficient to inhibit growth.

The E. coli cells incubated in tryptic soy broth containing 2.5 μ g of chloramphenicol per ml were diluted and inoculated onto nutrient agar and violet red bile agar to determine whether this concentration was sufficient to inhibit growth in the broth. On nutrient agar, the count of E. coli remained constant during the 3 hr incubation period, whereas the results observed on violet red bile agar showed a decrease in the viable count. The chloramphenicol appeared

to have a bacteriostatic effect when the treated cells were plated on nutrient agar, compared to an apparently bactericidal effect when plated on violet red bile agar. This is probably another expression of injury and might warrant further investigation.

The results for the experiment where 2.5 μ g of chloramphenicol were added/ml to the recovery medium (tryptic soy broth) are shown in Figure 11. When the cells from the recovery media, with and without chloramphenicol were plated on nutrient agar, typical and almost identical initial "recovery" curves were observed. However, after 6 hr at 37°C, the cells in the recovery medium without chloramphenicol started to grow, whereas no increase in cell numbers was observed in the recovery medium containing the 2.5 μ g chloramphenicol/ml. This was considered to be final proof that the initial changes in cell numbers observed on nutrient agar were a form of "recovery" and not growth. The cells from the control and chloramphenicol-containing recovery media were also plated on violet red bile agar. In the absence of chloramphenicol the expected recovery curve on violet red bile agar occurred. Contrary to expectation, the presence of chloramphenicol in the recovery medium inhibited the recovery of the heat-induced sensitivity to this selective agar.

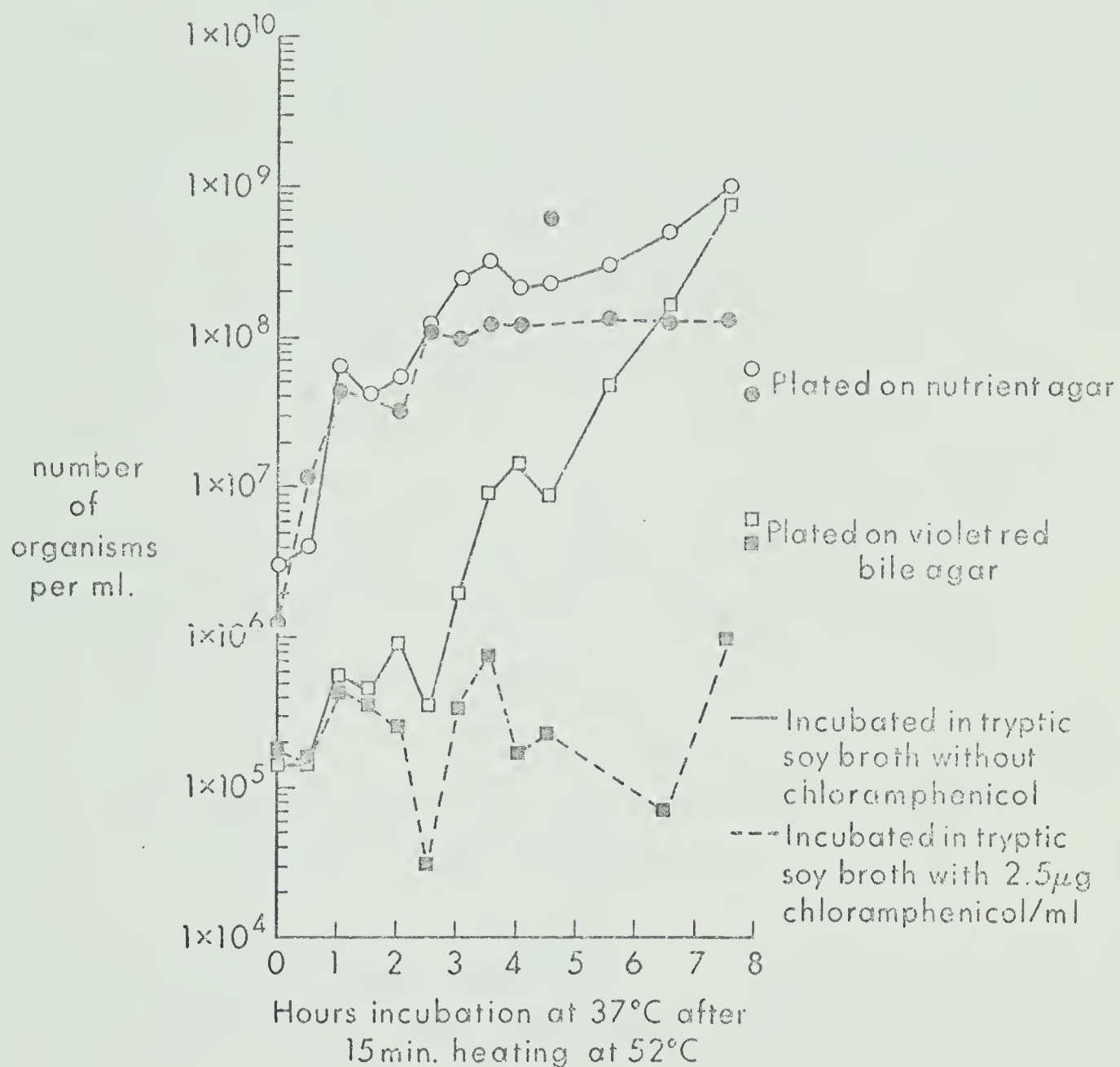


Figure 11. Recovery of heat injured *E. coli* SA211 with and without chloramphenicol added to the recovery medium.

The "recovery" of heated SA211 on the control medium during the first 3 to 3 1/2 hr of incubation at 37°C could possibly be attributed to a decrease in the nutritional requirements as suggested by Nelson (1943). However, the cause of this change was not studied and it might be due to other factors. The plateau in the control curve indicated that damage to the injured cells had been repaired, and that the expected lag period was now apparent. The recovery shown on the selective media was not complete at this time, and it was observed that the heat injured cells required approximately twice the time to recover their ability to grow on selective media compared to the control nutrient agar. The "recovery" curve established by nutrient agar indicated that the extent of death on the control medium might be exaggerated. Extrapolation of the 3 1/2 to 6 hr plateau back to the end of the heat treatment, indicated that only 25% "true" death occurred during the 15 min heating period, compared to 98% as indicated by the death curve (Figure 9).

Injury and recovery of *Salm. typhimurium*

The slopes of the thermal death curves of *Salm. typhimurium* strain 13311 in Figures 12 and 13 were plotted from the mean $D_{52^{\circ}\text{C}}$ values calculated from four trials. Also shown are the typical recovery curves for the four trials performed on each medium.

Nutrient agar was used in these trials as the control medium. However, Endo agar gave the slowest apparent death rate and therefore could be considered the control medium for this organism (Figures 12 and 13). This seemed reasonable since Endo agar is a differential

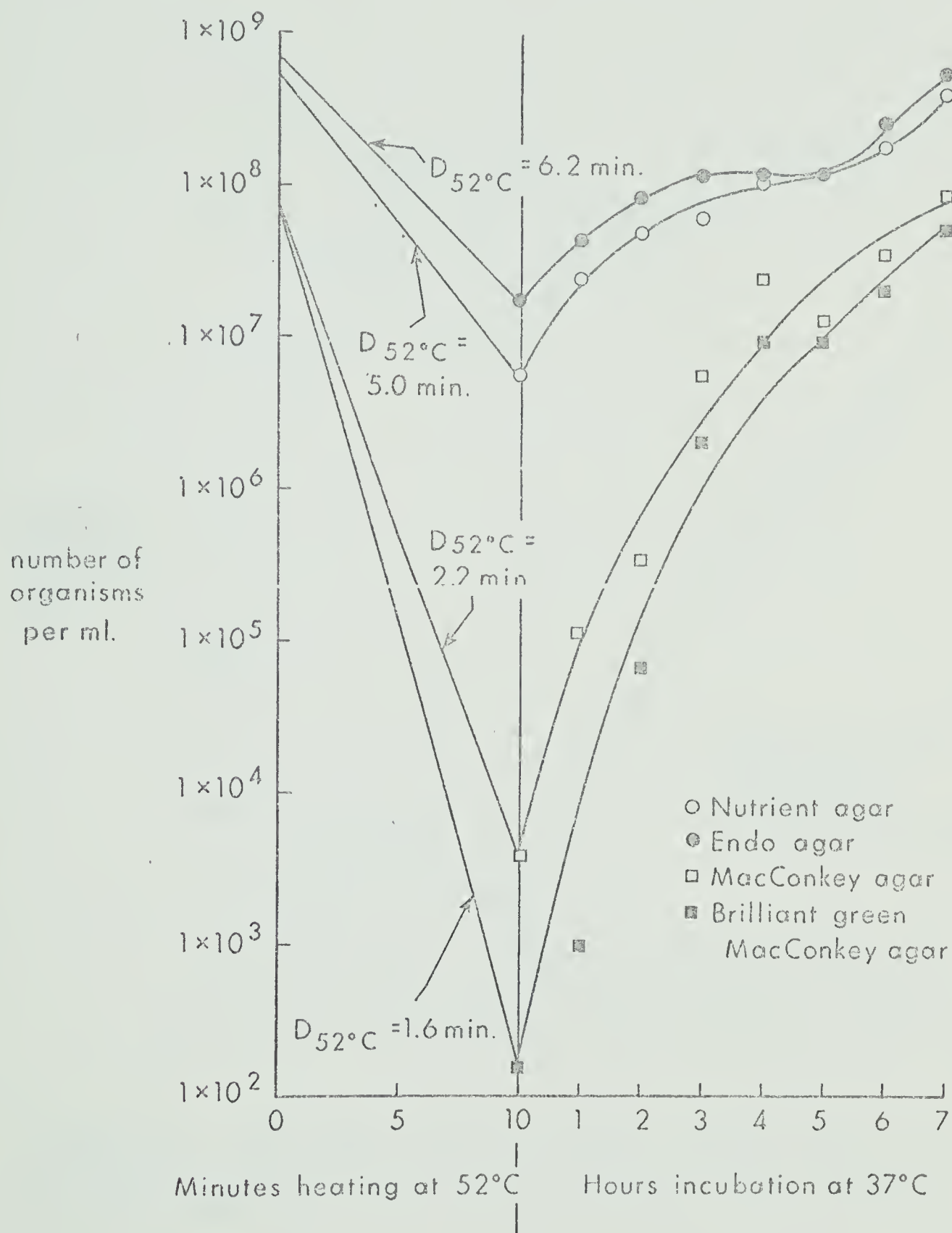


Figure 12. Mean death curves and typical recovery curves for *Salmonella typhimurium* 13311 obtained using surface plate technique.

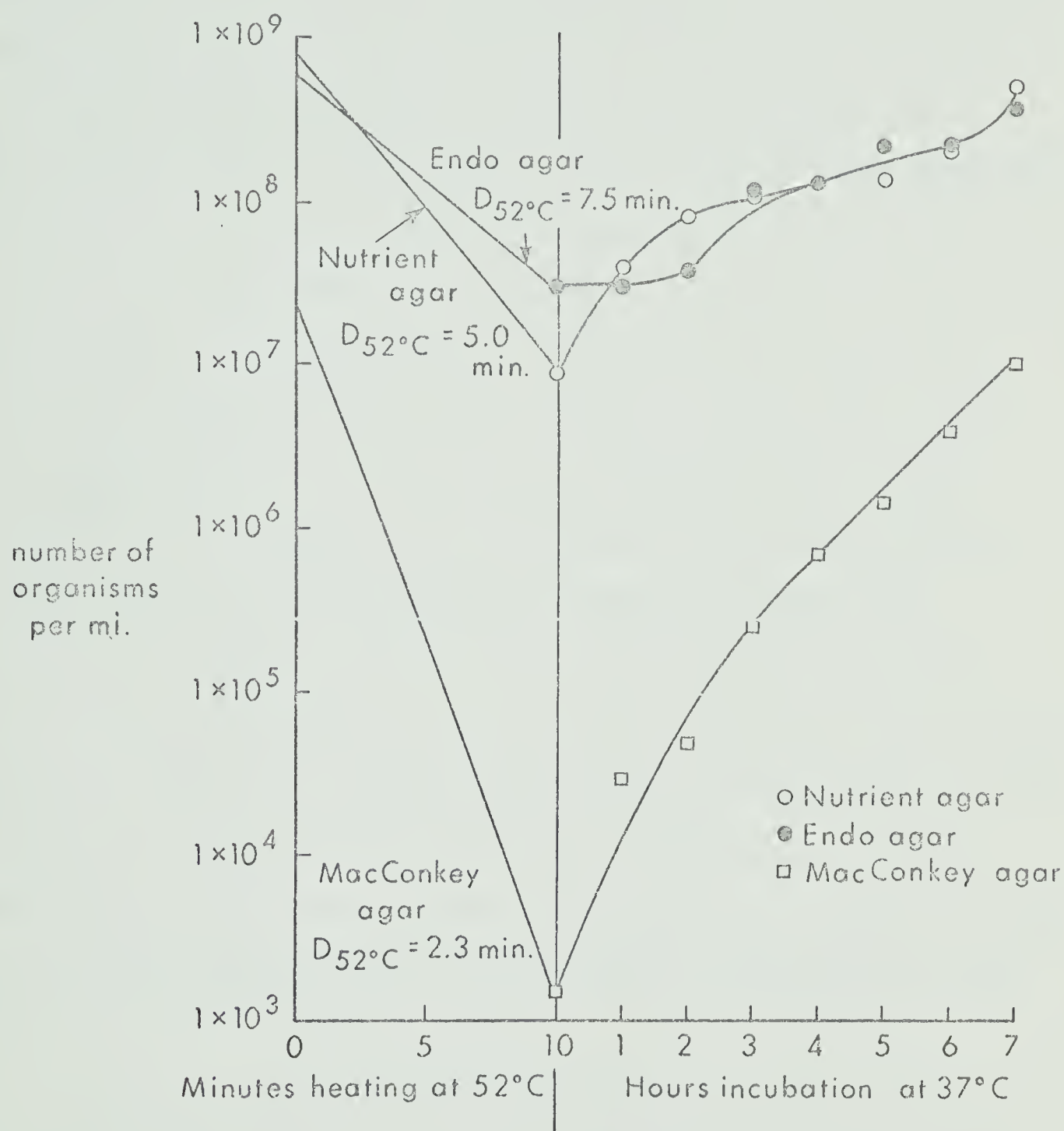


Figure 13. Mean death curves and typical recovery curves for *Salmonella typhimurium* 13311 obtained using pour plate technique.

rather than a selective medium and does not contain selective agents that might challenge the injured cells. Compared to Endo or nutrient agar, large amounts of injury ($> 99.95\%$) were shown when the cells were plated on MacConkey agar or brilliant green MacConkey agar. The extreme levels of injury noted on these selective media emphasized the necessity for injured cells to recover before they are plated on selective media. Since it is usually more important to detect the presence of salmonellae than to determine an exact count, an enrichment period usually precedes isolation on solid media. This enrichment process may allow some recovery and thus more accurate results would be obtained on the confirmatory solid selective media. During recovery the counts observed on nutrient and Endo agars increased approximately one log cycle. The fact that the initial rate of increase slowed down and that optical density did not increase with time (Figure 14) indicated that, similar to the observations with E. coli, the increased counts were due to "recovery", not growth. This initial increase was considered the same as that noted for E. coli and has been discussed fully in the section of "Recovery of E. coli" (p. 75).

Generally, the differences between pour and surface plate techniques were minimized and any differences could possibly be explained by the differences in oxygen tension. Nelson (1944) observed that thioglycollic acid in a synthetic basal medium caused large increases in counts of heat-treated E. coli. The higher

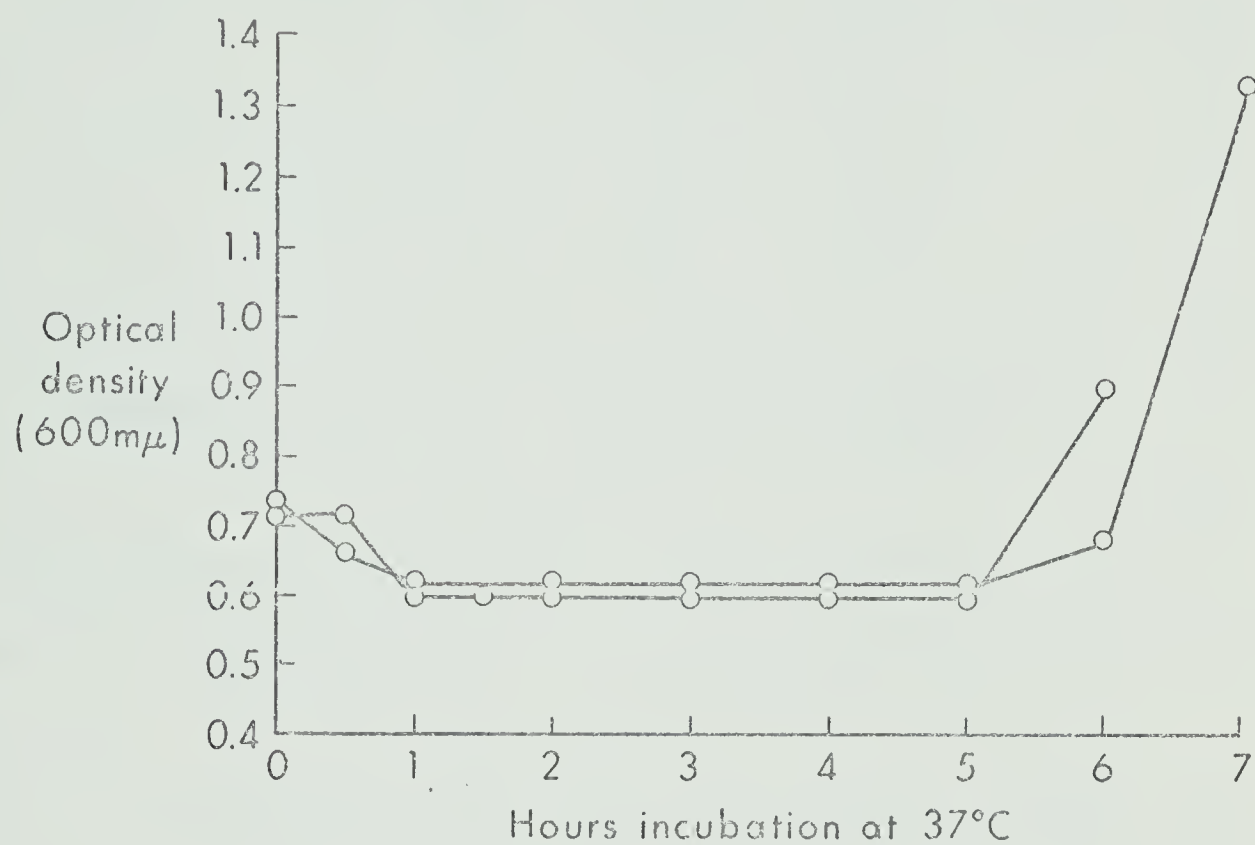


Figure 14. Optical density changes during recovery of Salmonella typhimurium 13311 in tryptic soy broth.
(Results of two trials.)

counts observed on nutrient agar when using the pour plate technique confirm the observations of Harries and Russell (1966) who noted "20% higher counts" of heated E. coli cultures in nutrient agar using the pour plate technique compared to surface plates.

Thatcher and Clark (1968) recommended the use of nonselective enrichment broths in the isolation of salmonellae when the test food has undergone a process that may "impart a semidormant or 'weakened' state" to any salmonellae cells present. To observe the effect of various selective enrichment broths on heat-treated (52°C for 10 min) Salm. typhimurium cells, 0.1 ml aliquots of a serial dilution were inoculated in triplicate into nonselective and selective enrichment broths (p. 37). Growth after 24 hr and 48 hr incubation at 37°C in these broths was verified where necessary by streaking on nutrient agar plates. The results generally showed little difference in growth of unheated cells after 24 and 48 hr incubation (Figure 15). From this it was evident that 24 hr enrichment of unheated cells was adequate. Perhaps it would be valid to use the shorter enrichment period as recommended for some of these broths. Enrichment of heat-treated salmonellae, using tetrathionate, selenite, and TT broths. showed more growth after 48 hr than after 24 hr incubation. With the emphasis in control laboratories on rapid results, it is important to consider whether the increased accuracy that can be obtained with the added time of incubation is warranted in the salmonellae detection test.

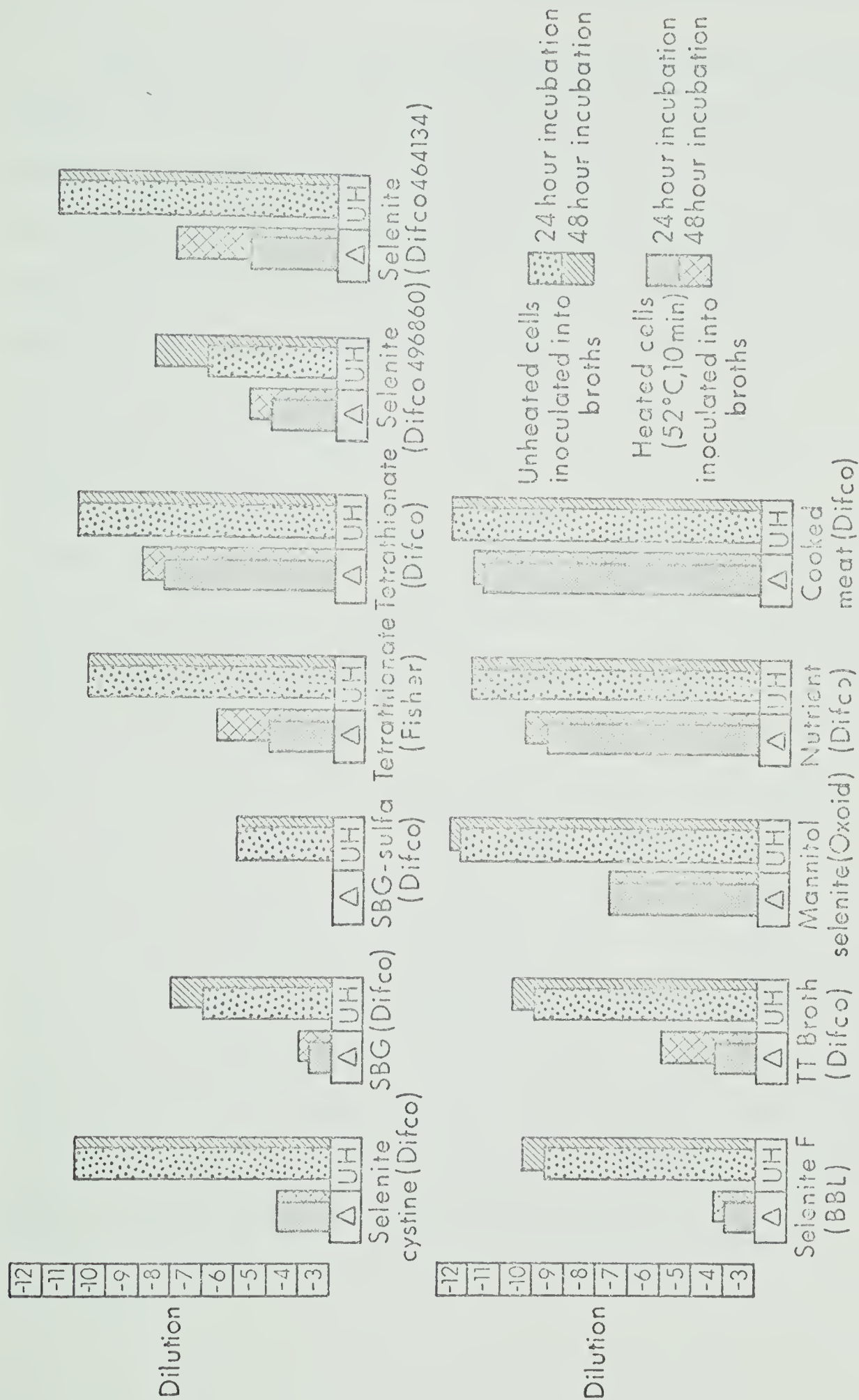


Figure 15. Dilutions of heated and unheated *Salmonella typhimurium* 13311

showing growth in salmonellae enrichment broths.

The nutrient and cooked meat broths were used as the control media. With these media it was assumed that the difference between heated and unheated values represented the actual death that had occurred (Figure 15). Nutrient broth gave lower amounts of heated cell growth than cooked meat broth. This is possibly due to superior growth providing substances being supplied by the cooked meat broth. Using cooked meat broth as a measure of the actual death, it was noted that far more death was apparent for the same heated cells grown in the selective enrichment broths. SBG and SBG-sulfa broths showed extreme levels of injury of Salm. typhimurium. However, they were so selective that they inhibited growth of unheated cells. These results did not compare favorably with those of Stokes and Osborne (1955) and Osborne and Stokes (1955) who noted abundant growth of salmonellae from very small inocula in these broths.

The data in Figure 15 indicate that different batches of Difco selenite broth and BBL selenite F broth were compared, as well as Fisher and Difco tetrathionate broth. The results indicated marked differences in the concentration of heated or unheated cells capable of initiating growth in these media. While the Fisher and Difco tetrathionate broths differed considerably in their ability to support the growth of heated cells, the differences between Difco selenite 464134 and 496860 were sufficient to indicate caution when changing batch lots in a control laboratory.

Difco's tetrathionate and Oxoid's mannitol selenite broths gave the best results for the selective broths supporting the growth of the heat-treated cells, but neither of these broths compared favorably with the controls. The results certainly emphasized the recommendation of Thatcher and Clark (1968) that nonselective enrichment be used when testing foods that may contain injured salmonellae.

Influence of bile salts

Since bile salts or their purified salt (sodium desoxycholate) are used as the selective agent in most of the selective media for E. coli, the effect of bile salts on the death rate of E. coli was checked. Just as the sodium chloride of some selective media appeared to be the challenging agent for injured Staph. aureus cells (Busta and Jezeski, 1963), perhaps the bile salts applied the stress to the injured E. coli cells, inhibiting their growth. To study the effect of bile salts on injured E. coli, Bile Salts No. 3 (Difco) was added to sterile nutrient agar in a concentration of 1.5 g/liter; and violet red bile agar without Bile Salts No. 3 was used for comparison. These media, with standard nutrient agar and violet red bile agar as controls, were used in two heating trials. The death curves for SA603 plated on these media were linear during the 15 min heating period, so that D values could be calculated and compared for the purpose of this study.

Results for SA603 showed an appreciable increase in the apparent death rate when bile salts were added to nutrient agar (Table 16). Similarly, a decrease in the apparent death rate was observed when the bile salts were removed from violet red bile agar. The increased death rate noted when bile salts were added to the media indicated that bile salts were probably responsible for part of the challenge to the injured cells in the selective violet red bile agar. However, the comparative D values for nutrient agar with bile salts added and violet red bile agar were markedly different. This indicated that other factors in the violet red bile agar were affecting the level of injury observed. This observation was supported by the converse situation, where the bile salts were removed from violet red bile agar. In this instance, the comparative D values for nutrient agar and violet red bile without Bile Salts No. 3 were not identical. Again, the other constituents of the violet red bile agar appeared to be causing the injury of a portion of the heated cells to be expressed.

The thermal death curves observed on the modified and control media for E. coli SA211 are shown in Figure 16. D values were not calculated for comparative purposes, because the apparent death curves on the "selective" media were nonlinear curves within the 15 min heating period. The differences in the death curves were not sufficient to conclude that bile salts in the growth media affected the death curves for SA211. The death curves on nutrient agar and nutrient agar with bile salts added were almost parallel, within the limits of

Table 16 Apparent $D_{52^{\circ}\text{C}}$ values for
E. coli SA603 on four media

Media	$D_{52^{\circ}\text{C}}$
Nutrient agar	23.2 min
Nutrient agar with 0.15% Bile Salts No. 3	11.1 min
Violet red bile agar without Bile Salts No. 3	11.8 min
Violet red bile agar	3.8 min

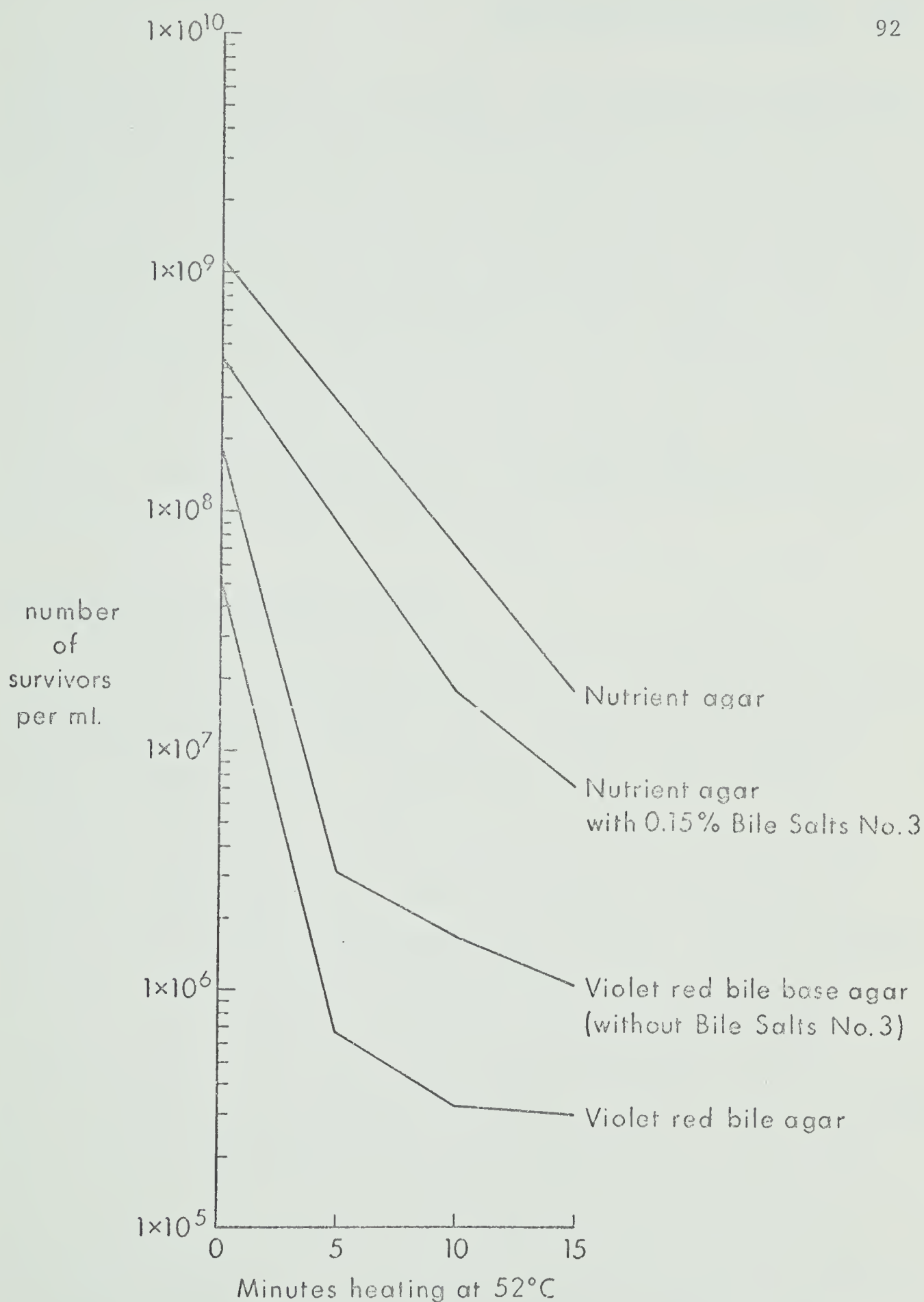


Figure 16. Thermal death curves for *E. coli* SA211 obtained using standard and modified nutrient and violet red bile agars. (Mean of two trials.)

nonlinearity occurring in the latter. This indicated that the bile salts were not restricting the growth of only the injured cells of SA211, but rather restricted a percentage of the total population. This confirmed the work of Wilson (1935) who noted a 25% lower count of E. coli in MacConkey medium compared to heart extract medium, and tested MacConkey medium without the bile salts. This gave a count almost exactly the same as that on heart extract agar. Wilson concluded that the inhibitory agent in MacConkey medium responsible for the lower counts was the bile salts.

Cottage cheese simulation studies

Sublethally-heated E. coli demonstrated an increased sensitivity to selective media but regained their tolerance when incubated in tryptic soy broth at 37°C. An environment that would simulate conditions encountered in cottage cheese manufacture was used to determine whether this heat injury and recovery phenomenon could occur in the cottage cheese manufacturing process. Sterile skimmilk (11% total solids) at pH 5 was used as the heating menstruum and recovery medium. Due to the protective effect of the skimmilk, it was necessary to use a heating temperature of 54°C to destroy about 90% of an E. coli population plated on nutrient agar. Two trials for each E. coli strain were run using nutrient agar as the control medium and MacConkey and violet red bile agars as selective media. For both E. coli SA603 and SA211 heat injury was shown on both selective media. The heating temperature of 54°C in 11% skimmilk resulted in death rates of E. coli SA603 similar to those noted on

heating at 52°C in tryptic soy broth (Table 10, p. 49). Mean $D_{54^{\circ}\text{C}}$ values for SA603 were: nutrient agar, 18.4 min; violet red bile agar, 3.8 min; and MacConkey agar, 5.1 min. The death curves observed for E. coli SA211 were similar to the curves obtained when cells were heated at 52°C in tryptic soy broth (Figure 5, p. 57).

The heat-injured cells, incubated in the heating menstruum at 37°C, after the sublethal heat treatment failed to recover their previous tolerance to the selective media (violet red bile and MacConkey agars) during the six-hour incubation (recovery) period. This indicated that under these conditions of medium and pH the recovery process was inhibited. For Staph. aureus (Iandolo and Ordal, 1966) and for Strep. faecalis (Clark, Witter and Ordal, 1968) it was shown that the recovery mechanism involved RNA synthesis. If conditions are unfavorable for RNA synthesis in unheated cells, then in the same conditions recovery of heat injured Staph. aureus or Strep. faecalis would not occur. Although lack of growth of normal cells does not necessarily imply that conditions would be unfavorable for recovery, it was decided to determine whether normal (unheated) cells of E. coli test strains could grow in skim milk at pH 5. Cultures were grown to $\text{OD } 1.0 \pm 0.04$, inoculated into sterile pH 5 skim milk and incubated at 37°C in shake cultures. Two trials for each strain were carried out and Figures 17 and 18 represent the typical results. It was observed that the counts of both strains on nutrient agar did not increase or decrease appreciably during incubation for 2 1/2 hr (Figures 17 and 18). The conditions in the

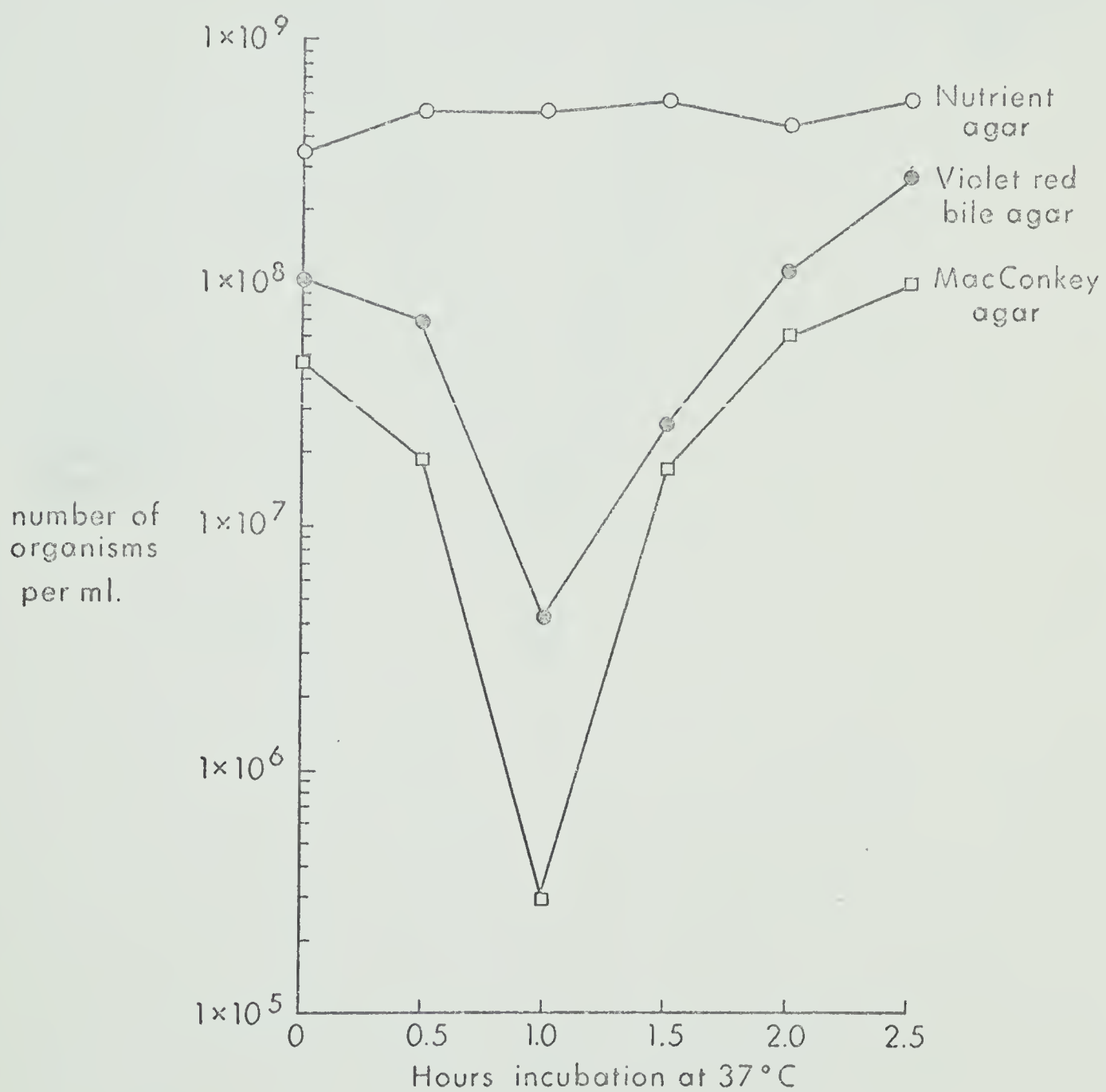


Figure 17. Change in counts of *E. coli* SA603 during lag phase of growth while incubated in skimmilk at pH 5.0.

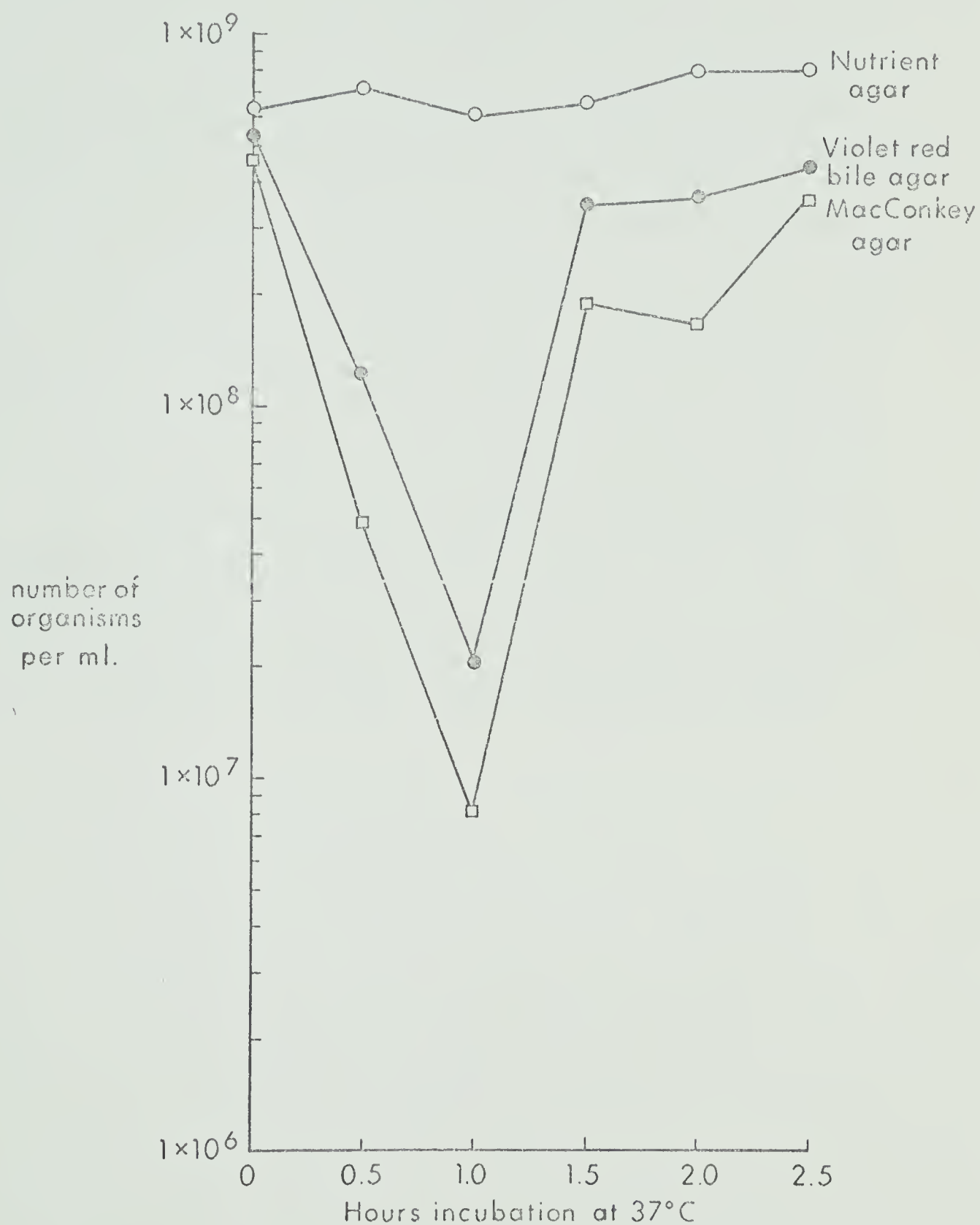


Figure 18. Change in counts of *E. coli* SA211 during lag phase of growth while incubated in skim milk at pH 5.0.

skimmilk did not destroy cells but apparently halted growth, causing an extended lag period. This possibly explains the lack of recovery of injured cells in this medium. While the viable count observed on nutrient agar did not change, a dramatic decrease in the counts was observed within the first hour on the selective media, violet red bile and MacConkey agars. The counts on these media rose sharply within the second hour and after this increase, were similar to those observed on these media at inoculation time. A similar decrease in the tolerance of exponentially growing cells when re-inoculated in tryptic soy broth was noted when they were plated on the selective media. It is suggested that these E. coli cells are susceptible to many forms of injury and that any form of 'shock' to the cells, such as transfer into fresh medium, low pH, antibiotics and sublethal heat treatment is sufficient to induce varying levels of cell injury.

CONCLUSIONS

The well established, though generally neglected fact, that the recommended selective media (violet red bile agar, MacConkey agar and the desoxycholate agars) for enumeration of coliforms gave lower counts than those observed on the nonselective control medium (nutrient agar) was confirmed in this study. Furthermore, it was observed that nutrient agar gave higher counts for coliforms than other nonselective media. The sublethal heat treatment of the E. coli organisms increased the difference between nutrient and selective media counts. It was this difference between the counts that was referred to as heat injury in these studies. The injury resulted in an increased sensitivity of a proportion of the heated cells to the selective media.

Sublethally heated E. coli SA603 showed an increased sensitivity to the selective agent, bile salts. However this inhibitor was not the only agent in the selective media for coliforms that presented a challenge to the injured cells inhibiting their growth. This was shown by the much faster death rate observed on violet red bile agar as compared to nutrient agar containing bile salts. While bile salts inhibited the growth of a percentage of the total population of E. coli SA211, it did not express heat injury for this strain.

The results indicated that while the selective media could not be relied on to support the growth of all the E. coli cells in an unheated culture, after sublethal heat treatment the ability of these selective media to support the growth of all of the cells present was drastically reduced. Where these media are used by food

and drug authorities to check the sanitary conditions under which foods are handled and produced, or to determine the safety of foods for human consumption, it is apparent that these media will frequently be inadequate for enumeration of the bacteria present.

Other expressions of heat "injury" were observed, that influence the suitability of these media for enumerating coliforms. With strain SA603, the apparent dilution inaccuracies in the experimental studies proved to be an inability of the heated cells at low concentrations to grow on the selective media although these concentrations were suitable for plating.

Unheated E. coli cells, plated on nutrient agar, gave colonies that could be counted after 24 hr incubation; under similar conditions after heat treatment, these colonies on nutrient agar were too small to enumerate at 24 hr. This was not the case with the selective media because the color of the colonies and the change in the color of the surrounding medium, made the colonies easy to identify, even after only 24 hr incubation.

From the results obtained with the broths it was concluded that BDG broth was the best selective medium for the presumptive test for coliforms after heat treatment. E C medium was shown to be unsatisfactory for use at 37°C and further study with E C medium at elevated temperatures is suggested.

The selective broths brilliant green bile 2% broth and MacConkey broth were as satisfactory as the nonselective broths (nutrient and lactose) in supporting the growth of unheated E. coli cells. However,

after heat treatment these selective broths were unsatisfactory for determining E. coli densities, because these media were unsuitable for supporting growth of the heat injured cells. Formate ricinoleate broth, which was specifically designed for the early production of gas by coliforms also proved unsatisfactory for the growth of E. coli after heat treatment.

A further expression of heat injury was noted with the broths. While most unheated cells inoculated into selective or nonselective broths showed maximum growth at 24 hr, after heat treatment it was necessary to incubate the tubes for 48 hr to obtain complete growth. As heating time was extended, the necessity for 48 hr incubation became more apparent.

After heat treatment there was a typical prolonged lag phase, and during this period at 37°C the injured cells recovered their ability to grow normally on the selective media. Recovery appeared to be temperature dependent, occurring at 20°C but not at 4.4°C. This recovery process affected the accuracy of the counts observed on the selective media, and therefore could assume considerable importance in public health microbiology and in quality control tests.

During the early stages of recovery of the E. coli strains, the counts obtained on nutrient agar increased and considerable effort was made to establish that the initial change in the counts on nutrient agar was recovery and not growth. It was concluded from the optical density results, the subsequent lag phase observed during recovery,

and the experiment incorporating chloramphenicol in the recovery medium, that the initial increase in count shown on nutrient agar was in fact recovery and not growth.

Yet another instance of injury was revealed in the chloramphenicol studies. After exposure to chloramphenicol, the numbers of cells growing on the selective media decreased, compared to those growing on nutrient agar. Throughout these studies, injury was seen to occur in many ways. This emphasized the necessity for caution in using the selective media for enumeration of coliforms in foods. Furthermore, the ability of the cells to recover from injury, placed even greater emphasis on the need for caution in interpreting coliform data.

Similar studies on Salm. typhimurium 13311 indicated that this organism was also subject to injury and recovery. Of particular concern was the limited efficiency of the solid media to support growth of the cells, especially after heat treatment. The broths, even those intended as enrichments for salmonellae, were also unsatisfactory. Differences were noted between batches of enrichment broths and between brands of the same broth. It is concluded that, as with E. coli, extreme caution is necessary in the enumeration of salmonellae on solid media and in the selection of enrichments for their isolation.

The simulated cottage cheese studies failed to confirm that recovery could be a source of coliforms in the final cottage cheese product. However, in these investigations, curd conditions were not

simulated, and probably only the pH was related closely to conditions that might be expected in the cheese. These studies were not exhaustive and might justify further reserach. Another avenue of study that is pertinent is the modification of selective media to support the growth of cells that have been injured as a result of exposure to some form of stress. This has virtually been achieved for Staph. aureus with the development of the egg-yolk containing media of Baird-Parker (1962) and Crisley, Peeler and Angelotti (1965).

In general, it appears that the media developed for the enumeration of coliforms are adequate when used with unheated cells. However, as soon as the coliforms are exposed to sublethal heat treatment the media are inadequate, and counts are subject to considerable error. With salmonellae however, many of the selective enrichment broths were unsatisfactory even with unheated cells of Salm. typhimurium. From these results it seems necessary that quality control laboratories should determine the efficiency of the enrichment broths for use under the conditions in their laboratories. Many factors may be involved, and appropriate media should be selected. From these studies it is apparent that considerable work is necessary on the development or modification of the media to be used for enumerating and detecting coliforms and salmonellae in foods.

- American Public Health Association. 1960. Standard methods for the examination of dairy products. 11th ed. Am. Public Health Assoc., New York.
- American Public Health Association. 1965. Standard methods for the examination of water and wastewater. 12th ed. Am. Public Health Assoc., New York.
- American Public Health Association. 1967. Standard methods for the examination of dairy products. 12th ed. Am. Public Health Assoc., New York.
- Baird-Parker, A. C. 1962. An improved diagnostic and selective medium for isolating coagulase-positive staphylococci. J. Appl. Bacteriol. 25:12.
- Bluhm, L. and Z. J. Ordal. 1969. Effect of sublethal heat on the metabolic activity of Staphylococcus aureus. J. Bacteriol. 97:140.
- Bonner, M. D. and L. G. Harmon. 1957. Characteristics of organisms contributing to spoilage in cottage cheese. J. Dairy Sci. 40:1599.
- Bradley, R. L., Jr. and L. G. Harmon. 1962. The effect of potassium sorbate on organisms commonly associated with cottage cheese spoilage. J. Dairy Sci. 44:1771.
- Busta, F. F. and J. J. Jezeski. 1963. Effect of sodium chloride concentration in an agar medium on growth of heat-shocked Staphylococcus aureus. Appl. Microbiol. 11:404.
- Cannon, R. Y. 1965. Effect of low levels of contamination on the shelf life of creamed cottage cheese. J. Dairy Sci. 48:6.
- Carreira, D. F. C., L. F. L. Clegg, P. A. Clough, C. C. Thiel, D. N. Akam, M. Gruber, and E. Hiron. 1955. The effect of steam, hypochlorite and caustic soda used for treating direct-to-can milking equipment, on the bacteriological quality and flora of milk. J. Dairy Res. 22:166.
- Clark, C. W., L. D. Witter and Z. J. Ordal. 1968. Thermal injury and recovery of Streptococcus faecalis. Appl. Microbiol. 16:1764.

- Clark, P. C. 1968. The influence of selective media on the enumeration of heat-treated Staphylococcus aureus. M.Sc. thesis, University of Natal, Pietermaritzburg, South Africa.
- Collins, E. B. 1961. Resistance of certain bacteria to cottage cheese cooking procedures. J. Dairy Sci. 44:1989.
- Crisley, F. D., J. T. Peeler and R. Angelotti. 1965. Comparative evaluation of five selective and differential media for the detection and enumeration of coagulase-positive staphylococci in foods. Appl. Microbiol. 13:140.
- Dept. of National Health and Welfare. 1954. The food and drug act and regulations with amendments to October 1, 1968. Queens Printer and Controller of Stationery, Ottawa. 1968.
- Difco Laboratories. 1953. Difco manual of dehydrated culture media and reagents for microbiological and clinical laboratory procedures. Difco Laboratories, Inc., Detroit, Michigan.
- El-Bisi, H. M. and Z. J. Ordal. 1956. The effect of certain sporulation conditions on the thermal death rate of Bacillus coagulans var. thermoacidurans. J. Bacteriol. 71:1.
- Emmons, D. B. 1963. Recent research in the manufacture of cottage cheese. Part II. Dairy Sci. Abstr. 25:175.
- Geminder, J. 1959. Extending shelf life of creamed cottage cheese. Milk Dealer 48(4):44.
- Hajna, A. A. and S. R. Damon. 1955. New method for detection of coliform organisms. J. Amer. Water Works Assoc. 47:631.
- Harmon, L. G. 1963. Cottage cheese problems in production and sanitation. J. Milk and Food Technol. 26(3):86.
- Harmon, L. G. and C. K. Smith. 1956. The influence of environment and processing on spoilage organisms in cottage cheese. J. Milk and Fd. Tech. 19:252.
- Harper, W. J. and H. E. Randolph. 1965. Microbiological quality and keeping properties of cottage cheese. Milk Dealer 55(2):48.
- Harries, D. and A. D. Russell. 1966. The revival of heat-damaged Escherichia coli. Experientia 22:803.
- Harris, N. D. 1963. The influence of the recovery medium and the incubation temperature on the survival of damaged bacteria. J. Appl. Bacteriol. 26:287.

- Heather, C. D., and W. C. Van der Zant. 1957a. Effect of the plating medium on the survival of heat-treated cells of Pseudomonas fluorescens. Food Res. 22:164.
- Heather, C. D., and C. Vanderzant. 1957b. Effects of temperature and time of incubating and pH of plating medium on enumerating heat-treated psychophilic bacteria. J. Dairy Sci. 40:1079.
- Hershey, A. D. 1939. Factors limiting bacterial growth. VII. Respiration and growth properties of Escherichia coli surviving sublethal temperatures. J. Bacteriol. 38:563.
- Hollaender, A. and B. M. Duggar. 1938. The effects of sublethal doses of monochromatic ultraviolet radiation on the growth properties of bacteria. J. Bacteriol. 36:17.
- Huang, Ellen Fen-Lan. 1969. The use of certain microbial inhibitors to increase the keeping quality of cottage cheese. M.Sc. thesis, University of Alberta, Edmonton, Canada.
- Iandolo, J. J. and Z. J. Ordal. 1966. Repair of thermal injury of Staphylococcus aureus. J. Bacteriol. 91:134.
- Jackson, H. 1963. 1963. A study of the bacteria in raw bulk tank milk. Ph.D. thesis, University of Alberta, Edmonton, Canada.
- Jackson, H., and M. Woodbine. 1963. The effect of sublethal heat treatment on the growth of Staphylococcus aureus. J. Appl. Bacteriol. 26:152.
- Kaufmann, O. W., L. G. Harmon, O. C. Pailthorp, and I. J. Pflug. 1959. Effect of heat treatment on the growth of surviving cells. J. Bacteriol. 78:834.
- Lawton, W. C. and F. E. Nelson. 1955. Influence of sublethal treatment with heat or chlorine on the growth of psychophilic bacteria. J. Dairy Sci. 38:380.
- Lyons, R. P. and W. L. Mallman. 1954. A bacteriological study of cottage cheese with particular reference to public health hazard. J. Milk and Food Technol. 17:372.
- Martin, W. H., J. D. Foltz, and W. D. Rutz. 1960. A survey of cottage cheese quality. J. Milk and Food Technol. 23:306.
- Mather, D. W., and F. J. Babel. 1959. Inhibition of certain types of bacterial spoilage in creamed cottage cheese by the use of a creaming mixture prepared with Streptococcus citrovorus. J. Dairy Sci. 42:1917.

- Ministry of Health. 1939. Reports on public health and medical subjects. No. 71. The bacteriological examination of water supplies. His Majesty's Stationery Office, London.
- Nelson, F. E. 1943. Factors which influence the growth of heat-treated bacteria. I. A comparison of four agar media. *Bacteriol.* 45:395.
- Nelson, F. E. 1944. Factors which influence the growth of heat-treated bacteria. II. Further studies on media. *J. Bacteriol.* 48:473.
- Nelson, F. E. 1956. The influence of the pH of the plating medium upon enumeration of heat-treated bacteria. *Bacteriol. Proc.* 1956:40.
- Olson, H. C. and O. D. Ball. 1957. Control of bacterial spoilage in cottage cheese. *J. Dairy Sci.* 40:1389.
- Osborne, W. W. and J. L. Stokes. 1955. A modified selenite brilliant green medium for the isolation of Salmonella from egg products. *Appl. Microbiol.* 3:295.
- Overcast, W. W., and J. D. Skean. 1958. Cottage cheese--needs carefully controlled manufacturing and marketing practices. *Tenn. Farm and Home Sci. Progress Report* #27.
- Overcast, W. W., and J. V. Britton. 1959. A study of the microbial flora of cottage cheese during storage. (Abstract) *J. Dairy Sci.* 42:910.
- Overcast, W. W., J. D. Skean, and J. V. Britton. 1961. Coliform bacteria in cottage cheese. *J. Dairy Sci.* 44:970.
- Parker, R. B., V. N. Smith and P. R. Elliker. 1951. Bacteria associated with a gelatinous or slimy curd defect of cottage cheese. *J. Dairy Sci.* 34:887.
- Perry, C. A. and A. A. Hajna. 1944. Further evaluation of F C medium for the isolation of coliform bacteria and Escherichia coli. *A. J. Pub. Health* 34:735.
- Russell, A. D., and D. Harries. 1968. Factors influencing the survival and revival of heat-treated E. coli. *Appl. Microbiol.* 16:335.
- Skelton, W. R. and L. G. Harmon. 1964. Growth rate of coliform organisms in cottage cheese and reconstituted nonfat dry milk. *J. Milk and Food Technol.* 27:197.
- Smillie, K. W. 1969. Statpack 2: An APL statistical package. 2nd ed. Department of Computing Science, University of Alberta. Publication No. 17.

- Stiles, M. E. 1963. Thermal inactivation and injury of Staphylococcus aureus. Ph.D. thesis, University of Illinois.
- Stiles, M. E., and D. Witter. 1965. Thermal inactivation, heat injury, and recovery of Staphylococcus aureus. J. Dairy Sci. 48:677.
- Strange, R. E. and M. Shon. 1964. Effects of thermal stress on viability and ribonucleic acid of Aerobacter aerogenes in aqueous suspension. J. Gen. Microbiol. 34:99.
- Stokes, J. L., and W. W. Osborne. 1955. A selenite brilliant green medium for the isolation of Salmonella. Appl. Microbiol. 3:217.
- Thatcher, F. S., and D. S. Clark. 1968. Microorganisms in Foods: Their significance and methods of enumeration. University of Toronto Press, Canada.
- Tuckey, S. L. 1959. Problems in cottage cheese production for 1959. J. Dairy Sci. 42:1246.
- Wilson, G. S. 1935. The bacteriological grading of milk. His Majesty's Stationery Office, London.

Appendix 1 Changes in the coliform content
of cottage cheese during manufacture
[Number (and percentage) of samples]

Dairy	Sample	Coliforms per g or per ml				
		<1	1 - 10	11 - 100	101-1000	>1000
A	Skimmilk	5(62.5)	1(12.5)	2(25.0)		
	Starter	8(100)				
	Cutting	5(71.4)	2(28.6)			
	Draining	7(87.5)	1(12.5)			
	Dry curd	7(7.8)	1(11.1)	1(11.1)		
	Dressing	2(22.2)	3(33.3)	4(44.4)		
	Creamed curd	2(25.0)	2(25.0)	3(37.5)	1(25.0)	
	Packed product	1(11.1)	2(22.2)	5(55.6)	1(11.1)	
B	Skimmilk	8(80.0)	2(20.0)			
	Starter	9(90.0)		1(10.0)		
	Cutting	10(100)				
	Draining	10(100)				
	Dry curd	9(90.0)		1(10.0)		
	Dressing	9(90.0)			1(10.0)	
	Creamed curd	8(80.0)	1(10.0)		1(10.0)	
	Packed product	4(40.0)	5(50.0)		1(10.0)	
C	Skimmilk		1(7.1)	5(35.7)	8(57.1)	
	Starter	13(100)				
	Cutting	1(8.3)		4(33.3)	6(50.0)	1(8.3)
	Draining	11(78.5)	1(7.1)	2(14.3)		
	Dry curd	10(71.4)	2(14.3)	2(14.3)		
	Dressing	12(92.3)	1(7.7)			
	Creamed curd	7(50.0)	1(7.1)	6(42.8)		
	Packed product	6(42.8)		7(50.0)	1(7.1)	
D	Skimmilk	4(25.0)	5(31.2)	3(18.8)	3(18.8)	1(6.2)
	Starter	13(100)				
	Cutting	3(18.8)	3(18.8)	5(31.2)	2(12.5)	3(18.8)
	Draining	13(86.7)	1(6.7)		1(6.7)	
	Dry curd	15(93.8)	1(6.2)			
	Dressing	3(23.1)	5(38.4)	3(23.1)	1(7.7)	1(7.7)
	Creamed curd	10(66.7)	3(20.0)		1(6.7)	1(6.7)
	Packed product	5(31.2)	3(18.8)	5(31.2)	2(12.5)	1(6.2)

Appendix 2 Changes in the bacterial content
of cottage cheese during manufacture

[Number (and percentage) of samples]

Dairy	Sample	Standard plate count per ml or per g						
		10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶	10 ⁷	10 ⁸
A	Skimmilk		4(80.0)		1(20.0)			
	Cutting			1(20.0)	4(80.0)			
	Draining		2(33.3)	3(50.0)	1(16.7)			
	Dry curd		5(83.3)	1(16.7)				
	Dressing	1(16.7)	5(83.3)					
	Creamed curd	5(83.3)	1(16.7)					
	Packed product	4(66.7)	2(33.3)					
B	Skimmilk				1(10.0)	3(30.0)	6(60.0)	
	Cutting				1(10.0)	2(20.0)	5(50.0)	2(20.0)
	Draining			4(40.0)	6(60.0)			
	Dry curd			4(40.0)	6(60.0)			
	Dressing	9(90.0)		1(10.0)				
	Creamed curd			3(30.0)	7(70.0)			
	Packed product			3(30.0)	7(70.0)			
C	Skimmilk					2(14.3)	10(71.4)	2(14.3)
	Cutting				1(8.3)	1(8.3)		10(83.3)
	Draining	1(7.1)	6(42.8)	4(28.6)	2(14.3)			1(7.1)
	Dry curd	2(15.3)	3(23.1)	6(46.2)	2(15.3)			
	Dressing	3(23.1)	8(61.5)	1(7.7)	1(7.7)			
	Creamed curd	1(7.1)	5(35.7)	6(42.8)	2(14.3)			
	Packed product	1(7.1)	4(28.6)	7(50.0)	2(14.3)			
D	Skimmilk		1(25.0)		3(75.0)			
	Cutting			1(25.0)	1(25.0)	2(50.0)		
	Draining		1(25.0)	1(25.0)	2(50.0)			
	Dry curd		1(25.0)	3(75.0)				
	Dressing		1(25.0)	1(25.0)		2(50.0)		
	Creamed curd		1(25.0)	2(50.0)	1(25.0)			
	Packed product		1(25.0)	2(50.0)	1(25.0)			

Appendix 3 Changes in the psychrophilic microbial content
of cottage cheese during manufacture

[Number (and percentage) of samples]

Dairy	Sample	Psychrophiles per g or per ml				
		< 1	1-10	11-100	101-1000	> 1000
A	Skimmilk		5(100)			
	Cutting		3(60.0)	1(20.0)	1(20.0)	
	Draining		5(83.3)	1(16.7)		
	Dry curd		5(83.3)	1(16.7)		
	Dressing		4(80.0)	1(20.0)		
	Creamed curd		4(66.7)	2(33.3)		
	Packed product		4(66.7)	2(33.3)		
B	Skimmilk		8(80.0)	2(20.0)		
	Cutting		7(70.0)	2(20.0)	1(10.0)	
	Draining		6(60.0)	4(40.0)		
	Dry curd		6(60.0)	4(40.0)		
	Dressing		7(70.0)	3(30.0)		
	Creamed curd		4(40.0)	5(50.0)	1(10.0)	
	Packed product		3(30.0)	7(70.0)		
C	Skimmilk		7(63.6)	4(36.4)		
	Cutting		6(54.5)	3(27.3)		
	Draining		6(54.5)	5(45.4)		
	Dry curd		9(81.8)	2(18.2)		
	Dressing		9(81.8)	2(18.2)		
	Creamed curd		4(36.4)	6(54.5)	1(9.1)	
	Packed product		7(63.6)	2(18.2)	2(18.2)	
D	Skimmilk				1(25.0)	3(75.0)
	Cutting		1(25.0)		2(50.0)	1(25.0)
	Draining		1(25.0)	1(25.0)	2(50.0)	
	Dry curd		1(25.0)		1(25.0)	2(50.0)
	Dressing				2(50.0)	2(50.0)
	Creamed curd		1(25.0)		1(25.0)	2(50.0)
	Packed product		1(25.0)		1(25.0)	2(50.0)

Appendix 4 Changes in the coliform content
of cottage cheese during storage at 4.4°C

[Number (and percentage) of samples]

Dairy	Sample	<1	Coliforms per g or per ml			
			1-10	11-100	101-1000	>1000
A	At packing		2(40.0)	3(60.0)		
	7 to 10 days		2(40.0)	3(60.0)		
	14 to 17 days		1(20.0)	3(60.0)	1(20.0)	
B	At packing	3(33.3)	5(55.6)		1(11.1)	
	7 to 10 days	1(11.1)	4(44.4)	1(11.1)	1(11.1)	
	14 to 17 days		7(77.8)			2(22.2)
C	At packing	2(33.3)		3(50.0)	1(16.7)	
	7 to 10 days		2(33.3)	3(50.0)	1(16.7)	
	14 to 17 days	1(16.7)	2(33.3)	3(50.0)		

Appendix 5 Changes in the bacterial content
of cottage cheese during storage at 4.4°C

[Number (and percentage) of samples]

Dairy	Sample	Standard plate count per g or per ml				
		$\leq 10^3$	10^4	10^5	10^6	$\geq 10^7$
A	At packing	3(60.0)	2(40.0)			
	7 to 10 days	1(20.0)		2(40.0)	1(20.0)	1(20.0)
	14 to 17 days			1(20.0)	1(20.0)	3(60.0)
B	At packing		2(22.2)	7(77.8)		
	7 to 10 days		3(33.3)	4(44.4)	1(11.1)	1(11.1)
	14 to 17 days			2(22.2)	4(44.4)	3(33.3)
C	At packing	2(33.3)	3(50.0)	1(16.7)		
	7 to 10 days	2(33.3)	3(50.0)	1(16.7)		
	14 to 17 days		2(33.3)	3(50.0)	1(16.7)	

Appendix 6 Changes in the psychrophilic microbial content
of cottage cheese during storage at 4.4°C

[Number (and percentage) of samples]

Dairy	Sample	Psychrophiles per g or per ml			
		≤ 10	11-100	101-1000	1001-10000 ≥ 10000
A	At packing	4(80.0)	1(20.0)		
	7 to 10 days	1(20.0)		1(20.0)	3(60.0)
	14 to 17 days				5(100)
B	At packing	3(37.5)	5(62.5)		
	7 to 10 days	2(25.0)	1(12.5)		3(37.5) 2(25.0)
	14 to 17 days			3(42.8)	4(57.1)
C	At packing	3(50.0)	1(16.7)	2(33.3)	
	7 to 10 days	2(33.3)	3(50.0)	1(16.7)	
	14 to 17 days			2(50.0)	1(20.0) 2(40.0)

Appendix 7 Changes in the yeast and mold content
of cottage cheese during storage at 4.4°C

[Number (and percentage) of samples]

Dairy	Sample	Yeast and mold count per g or per ml				
		$\leq 10^2$	10^3	10^4	10^5	$\geq 10^6$
A	At packing	1(20.0)	4(80.0)			
	7 to 10 days		4(80.0)			1(20.0)
	14 to 17 days				3(60.0)	2(40.0)
B	At packing	1(14.3)	3(42.8)	2(28.6)	1(14.3)	
	7 to 10 days			3(42.8)	4(57.1)	
	14 to 17 days			2(25.0)	2(25.0)	4(50.0)
C	At packing	4(66.7)	2(33.3)			
	7 to 10 days	3(50.0)	1(16.7)	1(16.7)	1(16.7)	
	14 to 17 days		1(16.7)	3(50.0)	2(33.3)	

Appendix 8 Titratable acidity (expressed as per cent lactic acid)
at cutting and final cooking temperature of the samples
obtained from four commercial dairies in Edmonton

Sample	Titratable Acidity, %	Final Cooking Temperature, F	Sample	Titratable Acidity, %	Final Cooking Temperature, F
A1	0.46	139	C6	0.56	130
A2	0.47	138	C7	0.54	127
A3	0.46	139	C8	0.56	122
A4	0.46	135	C9	0.55	123
A5	0.49	139	C10	0.55	122
A6	0.44	137	C11	0.55	124
A7	0.58	138	C12	0.57	123
A8	0.56	138	C13	0.54	122
Average	0.485	137.7	Average	0.554	125.0
B1	0.55	124	D1	0.52	•
B2	0.50	124	D2	0.52	115
B3	0.50	124	D3	0.58	•
B4	0.50	124	D4	0.58	125
B5	0.50	124	D5	0.58	115
B6	0.50	124	D6	0.53	112
B7	0.50	124	D7	0.60	134
B8	0.50	124	D8	0.62	130
B9	0.50	124	D9	0.56	120
B10	0.50	125	D10	0.57	132
Average	0.505	124.1	D11	0.55	121
C1	0.55	122	D12	0.54	116
C2	0.56	124	D13	0.53	128
C3	0.55	130	D14	0.59	122
C4	0.56	128	D15	0.58	119
C5	0.57	135	D16	0.56	125
			Average	0.564	122.4

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